PRODUCTION AND APPLICATION OF SPECIFIC ANTIBODIES TO EQUINE TUMOR NECROSIS FACTOR: RELEVANCE TO EQUINE ENDOTOXEMIA

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1994

ACKNOWLEDGMENTS

I am eternally grateful to my family for their unconditional love, support, and encouragement--especially to my mother for teaching me to cherish and respect animals, my father for showing me the world, my sister for friendship, my grandmother for believing in me, and all animals that have provided companionship over the years. Heart-felt thanks go to Dr. Rob MacKay for giving me the chance to work on this project, and for help and guidance along the way. Thanks also go to members of my supervisory committee for their advice, to Dr. John Dankert for help during the early stages of this project, and to faculty members of the UF Large Animal Clinical Sciences Department for advice and support. I am grateful to Leslie Skelley for her technical support, also to Marcus Brandt, Doug Smith, Barb Sheerin, Kelly Merritt, Brett Rice, Karen Gillis, and Jim Burrow for their help. I am indebted to Linda Green and everyone at the UF Hybridoma Facility for their contributions to this project. Thanks go to Gail Curtis for providing horses. And special tribute goes to the miniature horses and other animals that were used during this project. I'd also like to acknowledge the Morris Animal Foundation for providing funding for this research.

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

PRODUCTION AND APPLICATION OF SPECIFIC ANTIBODIES TO EQUINE TUMOR NECROSIS FACTOR: RELEVANCE TO EQUINE ENDOTOXEMIA

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December 1994

Chairperson: Dr. Robert J. MacKay
Major Department: Comparative and Experimental Pathology

Equine (Eq) tumor necrosis factor alpha (TNF) was prepared from serum of horses given lipopolysaccharide (LPS) intravenously (IV) using immunoaffinity and anion-exchange techniques. Cytotoxic activity was recovered in a product shown by SDS-PAGE and Western blot analysis to contain a band at 17.6 kDa (Eq TNF). Specific activity (units (U) TNF/mg protein) was enriched from 70 U/mg to 1 X 10^6 U/mg. Enriched Eq TNF was used to produce murine monoclonal antibodies (mAb) with high specific activity for Eq TNF. Two mAb were epitope-mapped (peptide binding analysis) to sequences within areas of Eq TNF that were predicted to be highly antigenic (residues 63-69 and 109-118, respectively). To help define the role of TNF in Eq endotoxemia, anti-Eq TNF mAb or isotype-matched control mAb was given to adult miniature horses (5 per group) at dosage of 1.86 mg/kg, IV.

Ten minutes later, LPS (E. coli 055:B5), 0.25 μg/kg, was given to all horses by IV infusion. Clinical signs were monitored and blood was taken for determination of white blood cell (WBC) count, packed-cell volume (PCV), plasma total protein (TP) concentration, plasma TNF and interleukin 6 (IL-6) activity, and plasma concentrations of lactate and eicosanoid metabolites (thromboxane B_2 (TXB_2) and 6-keto-prostaglandin-$F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$). Data were analyzed by ANOVA and Tukey's Honest Significant Difference test for significant ($P < 0.05$) effect of treatment. Compared to horses given control mAb, horses given anti-TNF had significantly improved clinical response, higher WBC count, and lower; circulating TNF and IL-6 activity, and plasma concentration of lactate and 6-keto-$PGF_{1\alpha}$. Fever, respiratory rate, PCV, plasma TP concentration, and plasma TXB_2 concentration was not significantly different between groups. TNF plays an important role in the responses of horses to endotoxin.

CHAPTER 1
INTRODUCTION

Endotoxin is a lipopolysaccharide (LPS) component of gram-negative bacterial cell walls.[1,2] Upon entry into mammalian circulation, LPS interacts with host phagocytes, platelets, and endothelial cells, and triggers the release of endogenous mediators that are responsible for the ensuing pathology.[2] Epidemiologic studies in humans have shown that the incidence of mortality due to septic and endotoxic shock has dramatically increased since the 1930's, to become the leading cause of death in human intensive care units.[3,4] This has initiated intense effort to elucidate the pathogenesis of endotoxemia and to develop more effective therapies.

Endotoxemia has also been implicated in the pathophysiology of several significant equine diseases.[5,6,7,8] The extensive equine gut is host to a large gram-negative bacterial flora, and conditions which disrupt the mucosal barrier can result in systemic circulation of endotoxins.[5,9] Reported long term survival of equine patients subjected to colic surgery and bowel resection was below 30%.[10] In addition, septicemia due to gram-negative organisms is a leading cause of mortality among equine neonates.[11] In a

major study, only 26% of foals with bacterial septicemia attributable to gram-negative organisms survived, despite intensive care.[8] As in human beings, great effort has been spent trying to understand, develop experimental models of, and find new therapies for, the devastating effects of bacterial endotoxins in the horse.

The difficulty in effectively treating endotoxemic patients is not surprising considering the plethora of mediators produced during endotoxemia. These include eicosanoids, stress hormones, cytokines, platelet-activating factor (PAF), reactive oxygen species (ROS), coagulation factors, complement products, kinins, neutral proteases, myocardial depressant factor (MDF), and nitric oxide (NO).[2,4,12-15] The complex pattern of mediator release during endotoxemic shock has been called a "systemic inflammatory response syndrome" (SIRS) and explains why specific mediator blockade may not be effective clinically.[16] Preventing the interaction of LPS with host cells would appear to offer an obvious advantage in preventing SIRS; however, anti-LPS serotherapy has had mixed results in experimental and clinical settings.[17] The development of neutralizing antibodies capable of protecting against the wide variety of naturally occurring endotoxins has been an elusive objective.[17]

In hope of identifying new therapeutic targets, there has been great effort to identify mediators released early

during endotoxemia that are absolutely crucial to initiating and continuing the SIRS. The criteria used to identify these pivotal mediators of LPS effects include the following: a syndrome similar to endotoxemic shock is produced when the mediator is purified and given parentally to experimental subjects; circulating mediator activities are detected early during the course of experimental and clinical endotoxemia; mediator blockade confers significant protection against the effects of endotoxin administration.

All of these criteria have been fulfilled, and a vast body of clinical and experimental evidence has been collected, that supports the role of tumor necrosis factor alpha (TNF) as a pivotal mediator of LPS effects.[18] Tumor necrosis factor alpha is a secretory polypeptide produced mainly by macrophages in response to endotoxin.[18] In horses, this cytokine has been isolated, molecularly cloned, characterized, and detected in the serum of horses and foals with naturally occurring disease.[19-24]

The broad aim of this dissertation is to further elucidate the role of TNF in equine endotoxemia. The working hypothesis was TNF plays a significant role in the responses of horses to endotoxin. Four areas were addressed to pursue this aim. First, a strategy was developed to produce native Eq TNF in large quantities and separate it from other serum components (discussed in chapter 3). Second, the partially purified Eq TNF was used as an

immunogen to produce monoclonal antibodies against Eq TNF. The production of these antibodies, and the characterization of their activity for Eq TNF, TNF from other species, and specific decapeptides corresponding to amino acid sequences within Eq TNF, is discussed in chapter 4. Third, the monoclonal antibody with highest Eq TNF-neutralizing activity was tested to determine the role of TNF in clinical and hematologic responses of miniature horses given a sublethal dose of LPS (chapter 5). Fourth, the effects of TNF blockade on metabolic status and production of mediators in these miniature horses was examined by evaluating interleukin 6 (IL 6) activity, lactate concentration, and thromboxane A_2 and prostacyclin metabolite concentration in plasma samples collected during the study (discussed in chapter 6). These investigations supported the experimental hypothesis, and resulted in development of reagents of potential therapeutic value that can be used to explore mechanisms of endotoxemia in the horse.

CHAPTER 2
BACKGROUND AND LITERATURE REVIEW

Pathogenesis of Endotoxemia

Neuroendocrine Activation

One of the earliest events to occur during endotoxemia is LPS-induced activation of the neuroendocrine system.[25,26] This results in stimulation of the hypothalamo-pituitary-adrenal axes and release of counter-regulatory (stress) hormones (glucagon, adrenaline, and cortisol).[25-27] This, combined with a decreased tissue sensitivity to insulin, results in hypermetabolism and hypercatabolism with increased energy expenditure.[25,28] Sympathetic stimulation and catecholamine response initially provides some compensation for declining systemic blood pressure, but as shock ensues potent vasodilatory mediators render the vasculature unresponsive to catecholamine effect.[29]

Like many other host responses to LPS, neuroendocrine activation is beneficial on a short-term basis to produce energy and maintain organ viability. However, prolonged hypermetabolic responses in face of progressive shock leads to malnutrition with increased susceptibility to secondary bacterial invasion and mortality. It should be noted that

extensive interactions occur between the neuroendocrine and immune responses. For example, glucocorticoids can directly suppress the transcription, translation, and production of a variety of cytokine and pro-inflammatory mediators, and IL-1 can stimulate adrenocorticotropic hormone (ACTH) release from the pituitary.[30-32] Both TNF and IL-1 have been implicated as mediators of neuroendocrine activation.[33,34] Administration of TNF or IL-1 increases plasma concentration of glucagon, adrenocorticoids, and catecholamines.[35,36]

Mediators of Systemic Inflammatory Response Syndrome

Macrophage activation. Extensive macrophage activation and production of pro-inflammatory products is a central event in the pathogenesis of septic shock.[33,37,38] One of the most potent of these products is TNF. Macrophage TNF mRNA and protein secretion rise 100- and 10,000- fold, respectively, upon exposure to LPS.[39] Transcription occurs at a much lower level, indicative of post-transcriptional regulation.[39] Plasma TNF activity is maximal approximately 90 minutes (min) following intravenous (IV) endotoxin administration.[18] The diverse biological activities that TNF elicits are all mediated via binding to specific surface receptors present on many cell types.[19] Major inflammatory effects of this cytokine during endotoxemia include induction of a variety of other proinflammatory mediators (IL-1, IL-6, PAF), and stimulation of ROS production by

granulocytes.[12,18,39] The role of TNF in the pathophysiology of endotoxemia will be explored later in this chapter.

Additional important macrophage products elaborated during endotoxemia include IL-1, IL-6, and PAF.[2,12] A multitude of other pro-inflammatory substances are also produced, including various coagulation factors, colony stimulating factors, leukotrienes, ROS, plasminogen activators, prostaglandins, leukotrienes, proteases, and nitric oxide.[12,13,15]

Like TNF, IL-1 is produced early during the course of endotoxemia and has a broad spectrum of target cells.[40,41] Some of the effects attributed to IL-1 include fever, induction of hepatic acute-phase proteins (APP), production of IL-6, production of PAF from endothelial cells, and production of prostacyclin by endothelial and smooth muscle cells.[12,40,41] A variety of metabolic functions are also affected by IL-1 including glucose homeostasis and amino acid utilization by muscles.[34,40,41] Circulating IL-1 activity has correlated with outcome in patients with meningococcal sepsis, and treatment with IL-1 receptor antagonist has prevented lethality in rabbits given endotoxin.[42,43]

Interleukin 6 is a potent stimulator of APP. In response to LPS, maximal plasma activity occurs after TNF and IL-1.[12,44] These observations, and the fact that many APP have an immunosuppressive effect, has led to the proposal

that IL-6 has negative feedback actions within the LPS-induced cytokine network.[12]

Platelet activating factor is released by a variety of cell types, including activated macrophages, and exhibits potent platelet- and granulocyte-aggregating and degranulating effects.[45] High concentrations of PAF are present in animals given LPS, and administration of purified PAF causes hypotension, plasma extravasation, metabolic acidosis and death.[45,46,47] Other activities attributed to PAF include a direct cardiac depressing effect, and induction of vascular permeability and hypotension.[45] Significant protection against lethality was provided by PAF receptor antagonists in an animal model of septic shock.[48]

Neutrophil activation. Cytokines and inflammatory mediators released during septic shock induce expression of neutrophil surface adherence glycoproteins and promote adherence of neutrophils to the vascular endothelium.[49,50] Increased expression of neutrophil adhesion molecules may also be a direct effect of LPS.[50,51] In addition, inflammatory mediators upregulate expression of vascular endothelial adhesion molecules for granulocytes.[50,52,53] In this microenvironment, the adhered neutrophil releases cytotoxic factors (PAF, ROS, proteases, granule-associated enzymes) which directly damage the endothelium.[49,50,52,53]

Products of arachidonic acid metabolism. Inflammatory mediators produced during endotoxemia cause arachidonic acid

to be released from plasma membranes and metabolized via the cyclooxygenase, lipoxygenase, or epoxygenase pathways to generate eicosanoids.[54,55,56] Eicosanoids are produced in a cell-specific fashion and have profound influences on vasomotor tone, microvascular permeability, platelet aggregation, and cellular activation for mediator release.[54,56,58] Prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) have opposing effects on vascular tone and aggregation of platelets, and are the major eicosanoids associated with hemodynamic alterations seen after LPS administration.[54,58-60] The production and effects of PGI_2 and TXA_2 during equine endotoxemia will be explored later in this chapter and in chapter 6.

Nitric oxide production. Nitric oxide (NO) is a potent vasodilator and inhibitor of platelet aggregation.[15,61,62,63] Leukocyte-endothelial interactions are also reduced by NO exposure.[15,61,62,63] As such, constitutive NO plays an important role in maintaining microvasculature integrity.[61,64,66] However, during endotoxemic shock, activation of inducible NO synthetase causes massive and sustained production of NO.[15,64,67] Under these conditions, NO has been shown to be an important mediator of systemic vasodilatory and hypotensive responses.[15,64,67]

The Vascular Endothelium as a Target Organ

Damage to the vascular endothelium, thrombosis, and microvasculature failure, are largely responsible for LPS-induced organ failure and mortality.[68] Multiple factors have a role in pushing the normal hemostatic balance towards pro-coagulant activity.[12,68,69] If unchecked, consumptive coagulopathy can become disseminated, exhaust pro-coagulant substrates, and cause diffuse hemorrhage.[12,68,70]

LPS can activate complement via both the alternative and classical pathways.[12,71,72] Complement can cause endothelial damage by several mechanisms including enhanced LPS-induced platelet activation and adherence, induction of granulocyte-inflicted endothelial damage, and activation of monocytes for cytokine production.[12,72,73,74]

Alterations in hemostasis can directly impair vascular endothelial integrity as well as induce thrombosis. Increased vascular permeability causes extravasation of fluid and plasma proteins.[12,68,70] During endotoxemia, LPS can initiate the intrinsic clotting cascade via Hageman factor, and/or, in the presence of tissue factor, activate the extrinsic clotting cascade.[12] When exposed to LPS-induced cytokines such as TNF and IL-1, endothelial cells can exert pro-coagulant and anti-fibrinolytic activities with excess production of tissue factor and plasminogen activator inhibitor.[12,74-77]

Mechanisms of Tissue Damage During Endotoxemic Shock

Oxygen radical production by phagocytes has been implicated as a major cytotoxic response to LPS.[13,78] Upon activation, phagocytes undergo an "respiratory burst" characterized by increased O_2 consumption and ROS production.[13,78,79] Initially, superoxide radical is formed which can serve as an oxidant directly, or can generate more powerful reactive oxygen species (H_2O_2, O_2) or oxygen free radical ($OH^{\cdot}$).[13,78,79] Lipid peroxidation by oxygen radicals is directly cytotoxic.[13,78,79] Many oxidant products are chemotactic and recruit additional inflammatory cells to the area.[13,78,79] Elastases and other proteases (lysosomal granule enzymes) are released by activated granulocytes during LPS-induced inflammation, leading directly to connective tissue damage.[80]

At the cellular level, ischemia leads to anoxia, metabolic derangements, acidemia, impaired membrane pump functions, cellular and mitochondrial edema, and, finally, release of lysosomal enzymes and cell death.[81,82] Anoxia induces a shunt to anaerobic glucose metabolism with production of lactic and other metabolic acids.[81,82] Acidemia ensues and further impairs cellular functions.[81,82] With exhaustion of cellular energy-producing capabilities, loss of essential cell functions occur ultimately leading to cell death.[81,82]

Summary

Mortality during endotoxemia occurs as a result of tissue hypoxia, vascular lesions, and cellular death caused by inflammatory and vasoactive factors released from host cells. Macrophages, platelets, endothelial cells, granulocytes, and many other cells generate both cytokines, which upregulate mediator production, and distal inflammatory mediators. Much of this response is directed against the vascular endothelium, and results in a hypercoaguable state, microvasculature perturbances, tissue ischemia and hypoxia, multiple organ failure, and death.

It is evident that a complex biological network of cellular signals exists, designed to protect against invading pathogens. As with many physiological systems, inherent checks and balances exist to ensure adequate response but prevent unnecessary tissue damage. Disruption of this homeostasis results in pathology. A better understanding of the mechanisms leading to dysregulation of immune/inflammatory responses may identify new treatment strategies for use in endotoxemic patients.

Experimental Endotoxemia in Horses

Infusions of lethal doses of LPS (>100 μg/kg). Early studies on endotoxin infusion in horses were performed in the 1970s and 1980s on anesthetized or awake ponies, using doses (100 μg/kg) of LPS 100,000 times higher than a dose

later shown to produce pyrexia in horses (1 ng/kg).[83-94] Endotoxin was given IV or intraperitoneally (IP).[83-94] Not surprisingly, the adverse effects of LPS administration at these dosages were severe clinical, cardiovascular, metabolic, and hematologic responses.[83-94] In awake ponies given LPS IV, marked tachycardia, tachypnea, pyrexia, colic, sweating, recumbency (3 h duration), and prolonged anorexia and depression (4 days duration) were reported.[85] Survival rates for ponies given LPS IV at this dose ranged from 0% to 67%.[85,91,92,93] Awake and anesthetized ponies exhibited fever (1 - 2 C elevations) that was maximal around 4 hours after LPS was given.[83,84,85]

Hematologic responses recorded within 2 h after LPS was given were abrupt and severe neutropenia, marked elevation in hematocrit, and thrombocytopenia. Lymphopenia developed by 4 h, rebound neutrophilia by 8 h, and hyperfibrinogenemia by 24 h after LPS was given.[85,89,90,91,] Prolonged clotting times with significant increases in the activated partial thromboplastin time and thrombin time were also reported.[91]

Within 10 min following IV LPS administration in anesthetized ponies, elevations in pulmonary artery pressure, central venous pressure (CVP), and total peripheral vascular resistance were recorded, accompanied by decreases in mean systemic arterial pressures (SAP).[87,88,90,92,] These changes correlated with peak thromboxane production.[88,92] This phase was followed by decreased CVP and

sustained decreases of mean SAP.[88,92] Increased prostacyclin production correlated with systemic hypotension.[88,92] Other researchers reported low cardiac output, decreased venous return, mixed respiratory and metabolic acidosis, increased blood flow to the gastrointestinal tract, skin, and skeletal muscle, and decreased flow to the CNS, kidney, and liver.[87]

Metabolic disturbances following IV LPS administration included initial hyperglycemia (2 - 4 h post-LPS), followed by hypoglycemia, and elevated blood lactate concentration.[85,86,89,91,94] Serum enzyme changes included increases in creatinine phosphokinase, lactate dehydrogenase, sorbital dehydrogenase, β-glucuronidase, and intestinal and total phosphatase activities.[85,86,89,91] Elevated cortisol concentrations were also reported.[91]

In ponies given single or multiple intraperitoneal (IP) doses of LPS, the hematological and metabolic changes were similar to those reported for IV administration, although clinical signs were more gradual in onset.[85,93,94] The pyrexic response to IP LPS appeared to be more severe (2 - 3 elevations), and of longer duration then that produced in ponies given similar amounts of LPS by the IV route.[85] Horses given LPS by direct infusion into the hepatic portal vein at a rate of 1 μg/kg/h for 24 h became recumbent, neutropenic, and exhibited elevated fibrinogen and fibrin split products, and shortened recalcification tests and activated partial thromboplastin times.[95] The hepatic

portal vein infusion model is the only model where LPS administration to horses caused signs of acute laminitis--despite the common clinical observation that suspected endotoxemic horses often develop laminitis.

Infusion of sublethal doses of LPS (1 ng/kg - 10 μg/kg). Over the past decade, infusion studies in conscious horses and ponies were conducted with lower doses of LPS purported to more accurately reflect syndromes in clinical cases.[20,96-110] Intravenous administration of 10 μg LPS/kg to horses and ponies resulted in a well defined clinical response.[20,96-100] During the first 2 - 3 min after LPS was given, moderate tachypnea, tachycardia, respiratory alkalosis, and ataxia were seen.[20,96-100] Colic and passage of a small amount of non-formed feces consistently occurred within 60 min after LPS was given.[20,97,98] Fever developed within 3 h of LPS administration.[20,97,98] Prolonged lateral recumbency was not reported and all subjects recovered.[20,96-100] Neutropenia, thrombocytopenia, and elevated hematocrit were reported, similar to that seen in ponies given higher doses of LPS; however, elevations in hematocrit were less severe.[20,96-100] Infusion of 30 ng/kg endotoxin in horses produced mild clinical signs, fever, and failed to cause changes in prothrombin, activated partial thromboplastin, thrombin, whole blood recalcification times, or concentration of fibrin split products.[20,101,103-106] This dose

did produce a significant elevation in fibrinogen at 24 h post-LPS.[103]

Lactate reached maximum plasma concentration of 35 mg/dl in ponies given 10 μg/kg LPS, compared to 100 mg/dl in ponies given 100 μg/kg LPS.[91,97] Plasma eicosanoid metabolite (TXB_2 and 6-keto-$PGF_{1\alpha}$) responses were similar in horses given LPS dosages of either ≥ 100 μg/kg or 10 μg/kg.[88,92,98,100] In horses given 30 ng/kg LPS, the time-course of eicosanoid production was similar to that at higher doses; however, maximal concentration was lower.[103]

In anesthetized horses, an IV LPS infusion of 0.9 - 2.5 μg/kg raised pulmonary arterial pressures by 10 mmHg.[102] Changes in central venous pressure and mean arterial pressure were not significant at this dose.[102] In awake horses given a 60-minute infusion of LPS (total dose: 30 ng/kg), pulmonary arterial pressures increased significantly while cardiac output, systemic arterial pressure, total peripheral resistance, and right atrial pressure did not change.[104] This dose of LPS caused significant reduction of cecal arterial blood flow, as compared to increased splanchnic flow demonstrated in ponies given 200 μg/kg LPS.[87,104] Doses of LPS of 100 ng/kg and 30 ng/kg were both shown to significantly inhibit gastrointestinal tract motility.[87,105,109]

Recent studies in horses and foals have defined the TNF and IL-6 responses during experimental induction of

endotoxemia.[20,101,106,107,108,110] In horses given 30 ng/kg and 1000 ng/kg LPS, and foals given 0.5 μg/kg and 0.25 μg/kg LPS, serum TNF activity increased within 1 h after LPS was given, and remained elevated for 4 h. [20,101,107,110] The increased circulating TNF activity correlated with increases in heart rate and clinical abnormality scores.[101] Interleukin 6 activity was significantly elevated over baseline values in horses given 30 ng/kg and 1000 ng/kg, obtaining maximal activity within 3 h after LPS was given.[106] Foals also responded to LPS with elevations in IL-6 activity; however, maximal activity was present 2 h after LPS administration in this group.[108]

Effects of various treatments on endotoxemic horses. The effects of non-steroidal anti-inflammatory drugs and corticosteriods have been studied in lethal and sublethal models of equine endotoxemia.[20,80,87,89,90-94,97,98,100] Phenylbutazone was shown to significantly attenuate hemoconcentration, hyperglycemia, increases in blood lactate, and fever when given IV 30 min after a lethal dose of LPS was given IP.[80] Survival rate was not increased with phenylbutazone treatment.[80] When anesthetized ponies were treated with flunixin meglumine after a lethal dose of LPS was given IV, they maintained normal mean arterial blood pressure and blood flow to the CNS.[87] Endotoxin-associated decrease in blood pH and increase in lactate were attenuated with flunixin treatment.[89,99] Plasma concentration of

thromboxane and prostacyclin metabolites, and associated hemodynamic alterations, were significantly reduced by flunixin pretreatment.[87,92,94,98] Flunixin also reduced fluid shifts to the interstitial space and hemoconcentration associated with LPS administration.[90] In conscious ponies given a sublethal dose of LPS IV, flunixin pretreatment prevented the development of respiratory alkalosis, metabolic acidosis, arterial hypoxemia, and increased blood lactate concentration for a 90-minute period following LPS administration.[97] Pulmonary artery endothelial damage was attenuated when flunixin meglumine was given immediately before multiple IP doses of LPS.[93]

When flunixin meglumine, phenylbutazone, and a selective thromboxane synthetase inhibitor (TSI) were compared in a sublethal LPS model, flunixin meglumine and TSI prevented LPS-induced TXB_2 increase, while phenylbutazone was less efficient at blocking this response.[98] Both phenylbutazone and flunixin prevented the prostacyclin metabolite increases, while TSI had no effect on 6-keto-$PGF_{1\alpha}$.[98] Clinical signs were most attenuated in flunixin-treated ponies, while those given TSI were more severely affected than ponies given LPS alone.[98]

Dexamethasone treatment of ponies given LPS did not prevent hematologic or blood gas changes, but did attenuate plasma lactate increases and prolongation of clotting times.[91,92] When dexamethasone and prednisolone treatment was

compared to flunixin treatment in ponies given LPS, only flunixin treatment prolonged survival times, prevented hemoconcentration, and abrogated elevations in serum enzyme activities (creatine phosphokinase, lactate dehydrogenase, sorbitol dehydrogenase, β-glucuronidase, and intestinal and total phosphatases).[91] Dexamethasone and prednisolone treatment attenuated the prolonged clotting times and induced a faster return to baseline values of circulating neutrophils after LPS-induced neutropenia.[91] None of the treatments reduced blood lactate concentrations in this study.[91]

A number of other treatments have been examined in models of equine endotoxemia. The efficacy of antiserum to core-lipid A has been studied in experimentally-induced and naturally-occurring endotoxemia in horses and foals.[99,110-113] Early studies reported that core-lipid A antiserum lessened depression, colic, and anorexia during experimental E. coli endotoxemia, and improved clinical condition in horses with diseases associated with endotoxemia.[111,112] Subsequent studies failed to reproduce these results in either endotoxemic equine patients or horses given LPS.[99,110,113] One study documented increased severity of clinical disease and enhanced cytokine production in foals treated with core-lipid A antiserum, then given LPS.[110] In contrast, pretreatment with polymyxin B, an antibiotic that binds

lipid A, resulted in significantly lower circulating cytokine activity, rectal temperature, respiratory rate.[110]

The influence of ω-3 fatty acids (linseed oil) on the in vivo responses of horses to endotoxin was studied.[103] In contrast to decreased eicosanoid synthesis shown in vitro by blood cells from horses on this dietary regime, in vivo eicosanoid metabolite production after LPS administration was not different between horses on the linseed oil diet as compared to control diet.[103,114] The only significant in vivo effect of the ω-3 supplementation was longer clotting times, compared to those of control-fed horses, both before and after LPS administration.[103]

A specific PAF receptor antagonist was evaluated in a model of Eq endotoxemia, and was shown to block LPS-induced platelet aggregation in vitro.[115] Treatment with PAF receptor antagonist delayed, but did not prevent, the onset of fever, tachycardia, leukopenia, and lactic acidosis in horses given endotoxin.[115] Pretreatment with allopurinol in horses given LPS IP significantly reduced rises in xanthine oxidase, an enzyme that converts hypoxanthine to uric acid with formation of superoxide radicals during reperfusion injury.[116] Treatment with naloxone, a β-endorphin antagonist, was not successful in preventing LPS-associated blood flow or hemodynamic changes during LPS-infusion.[117] Hypertonic saline resuscitation had minor protective effects, as compared to infusion of an equivalent volume of

isotonic saline, in maintaining cardiac output and preventing increases in total peripheral vascular resistance in horses given endotoxin.[102] In horses given an infusion of 30 ng/kg LPS, α_2 adrenergic blockade with yohimbine prevented LPS-associated cecal hypoperfusion and ileus.[109]

Summary. Endotoxemia induced experimentally in horses can be divided into two distinct phases. During the first hour after LPS is given, TXA_2 is released rapidly into circulation. Tachypnea, tachycardia, increased PAP, hypoxemia, hypocapnia, and respiratory alkalosis occur concurrently. At higher dosage of LPS, cardiac output and mean arterial pressure is decreased, and central venous pressure is elevated. Blockade of TXA_2 production with flunixin reverses many of these changes. A rise in hematocrit occurs secondary to splenic contraction and circulating neutrophil count begins to decline abruptly. During this phase, horses appear anxious and may exhibit signs of colic.

After the acute phase, dose-related differences in the response to LPS become more apparent. At higher (lethal) doses, mean arterial pressure and cardiac output continue to decline, central venous pressure falls, and blood flow is directed away from vital organs because of pooling in the splanchnic circulation, skin, and muscle. Lower dosage of LPS does not produce changes in cardiac output or systemic hypotension, and blood flow to the gastrointestinal tract

may be decreased secondary to α_2 adrenergic stimulation. One hour after LPS is given, circulating TNF activity rises, reaching maximal activity at approximately 90 min, and remains elevated for 4 h. Plasma concentration of TXA_2 falls while those of PGI_2 rise to maximal concentration 2 h after LPS is given. A febrile response is produced at all doses, and temperature gradually climbs to maximum value by 3 - 4 h after LPS is given. A second tachycardic and tachypneic response occurs during this period of fever. Horses exhibit dose-dependant signs of colic, depression, anorexia, and recumbency. Interleukin 6 activity in plasma becomes significantly elevated 2 h after LPS is given. Dose-dependant increases of blood lactate concentration is evident by 2 h after LPS administration, and reflects a shift to anaerobic metabolism in oxygen-deprived tissues. As cellular damage occurs from ischemic insult, circulating cell-associated enzyme activities increase. After lethal dosage of LPS, hypotensive shock develops, producing progressively worsening metabolic acidosis, declining cardiac output and central venous pressure, continued derangement of metabolic function, reduced tissue perfusion, and death. If sublethal dosage of LPS is given, hypotensive shock does not develop, and clinical and hematological abnormalities are mostly resolved by 24 h.

Treatment with flunixin blocks the thromboxane and prostacyclin responses, and associated cardiovascular

disturbances, in horses given endotoxin, but does not reverse LPS-induced neutropenia, cytokine production, or coagulopathy, and only partially attenuates clinical signs and fever. The role of TNF in LPS-induced responses in the horse will be addressed in chapters 5 and 6. Other important mediators such as IL-1, nitric oxide, and PAF have not been studied in detail in equine endotoxemia.

Tumor Necrosis Factor Alpha and Endotoxemia

Isolation, production, and characterization of TNF. In the late 1800's, William Coley observed regression of tumors in some cancer patients with concurrent bacterial infections.[118] This observation was confirmed and the name "tumor necrosis factor" was coined in 1975 for an endotoxin-induced serum factor that caused necrosis of transplanted tumors in mice.[119] In 1985, a macrophage-derived factor induced by LPS was shown to inhibit lipase activity, and was called "cachectin" for its presumed role in cachexia of chronic disease.[120] Sequence analysis subsequently revealed TNF and cachectin to be the same protein.[121,122]

Murine TNF was purified from LPS-stimulated macrophages (RAW 264.7) using ion-exchange chromatography.[121] When subjected sodium dodecyl sulfate polyacrylamide gel electrophoresis, murine TNF possessed a molecular mass of 17 kDa.[121] Human TNF was isolated from tissue culture supernate of a phorbol ester-treated promeylomonocytic cell line (HL-

60) by sequential controlled-pore ion-exchange, and fast protein liquid chromatographic techniques.[123] The purified protein had specific cytotoxic activity for L929 cells of 10^8 U/mg and a molecular mass of 17 kDa.[123]

Genes encoding for TNF of human, horse, and many other species have been molecularly cloned and expressed.[22,124-127] The chromosomal location of human and murine TNF genes is within a region containing the major histocompatibility complex, and comprises 4 exons that code for a precursor product of 233 amino acids and a mature protein of 157 amino acids.[128] Crystallographic studies have shown that active TNF is trimeric.[129] Detailed structure-function analysis of TNF is presented in chapter 4.

The macrophage is the principal source of TNF *in vivo*. Stimulated macrophages produce TNF and drugs such as carrageenan, which are cytotoxic for macrophages, suppress TNF production *in vivo*.[38] Tumor necrosis factor alpha exerts its pleiotropic effects by binding to specific cell surface receptors distributed widely throughout the body.[130] Post-receptor mechanisms have not been clarified completely; however, transduced signals are partly controlled by G proteins, and result in activation of adenyl cyclase and phospholipase A_2 second messenger pathways.[130] Early evidence for a role of TNF in the pathogenesis of endotoxemia came from studies in which infusion of TNF was shown to cause an endotoxemic shock-like effect. Passive

immunization against TNF substantially mitigated lethal endotoxin effects, further supporting this role.[121,131]

Effects of TNF infusion. Infusion of TNF in humans, dogs, sheep, mice, and rats mimics effects seen during endotoxemia and septic shock.[35,132-137] Infusion of recombinant-derived human TNF (rHu TNF) in rats resulted in hypotension, hyperglycemia, lactic acidosis, shock and respiratory depression.[132,133] Mice reacted similarly to infusion of rHu TNF with hypothermia, hypoglycemia, lactic acidosis, elevated hematocrit, and neutropenia.[134] Administration of rHu TNF to dogs produced elevations in plasma catecholamine, cortisol, and glucagon concentration, and, with higher dosage, there was progressive hypotension, shock, and death.[35] In human beings, administration of rHu TNF produced fever, tachycardia, neutropenia, lymphopenia, stress hormone release, and activation of coagulation.[135,136] It was interesting to note that TNF could not be detected in serum after 6 h of TNF infusion, despite continued infusion of biologically active TNF--indicating probable induction of soluble TNF receptor activity.[135]

The effect of rHu TNF infusion on hemodynamics and eicosanoid synthesis was studied in anesthetized sheep compared to effects of LPS infusion.[137] Infusions of TNF and LPS both caused leukopenia and increased plasma 6-keto-$PGF_{1\alpha}$, but elevated plasma TXB_2 was seen only when LPS was given.[137] Profound systemic hypotension and vasodilation was

produced when TNF was given, while pulmonary arterial hypertension and gradual systemic hypotension without peripheral vasodilation occurred when LPS was given.[137] Meclofenamate (cyclooxygenase inhibitor) prevented increase in plasma prostacyclin metabolite concentration and early hypotension in sheep given rHu TNF, but did not affect hypotension developing from 4 to 6 h after TNF was given.[137]

Interpreting results from infusion studies is complicated because recombinant-derived TNF may be contaminated with small amounts of LPS. Several rodent studies have indicated that TNF alone does not induce lethality unless combined with small amounts of LPS.[138,139] Two reports indicated that IL-1 activity was not induced by TNF infusion in humans or mice, however endotoxin administration also failed to induce IL-1 activity in some studies.[134,135,140] Thromboxane production and the associated pulmonary arterial pressure increases were not induced by TNF infusion.[137] Infusion of TNF did reproduce fever, leukopenia, systemic hypotension, and metabolic changes associated with endotoxin administration.[35,132-137]

Correlation of circulating TNF activity with clinical disease. In a variety of clinical and experimental studies in human beings, primates, pigs, and mice, circulating TNF activity correlated with severity of disease and fatal outcome.[44,77,141-148] Additionally, increased circulating TNF

activity was been measured in non-surviving septic human patients after antibiotic therapy was initiated.[77]

Neutralization of TNF during endotoxemia. Prophylactic and therapeutic use of antibodies against TNF has been protective in many models of endotoxemia.[131,146,149-158] A murine monoclonal antibody (mAb) against Hu TNF (CB0006) is currently being evaluated in clinical trials of patients with sepsis syndrome.[160] These studies have provided the strongest support for the important role of TNF in the pathophysiology of endotoxemia.

Mice given polyclonal rabbit anti-mouse TNF (Mu TNF) were protected against lethal outcome during endotoxin challenge.[131] Rabbits treated with goat anti-rHu TNF were protected against systemic hypotension and lethal effect of LPS.[146] Febrile and leukocyte responses were not affected by anti-TNF treatment in these models. Mice treated with a TNF mAb prior to administration of E. coli LPS had less weight loss and anorexia, and attenuated IL-6 and APP production than control LPS-treated mice.[149] In baboons, pretreatment with Mu mAb against rHu TNF given prior to live E. coli infusion reduced circulating IL-1 and TNF activity and protected against shock, multiple organ failure, stress hormone release, and death.[150,151] In models of porcine sepsis due to P. aeruginosa or E. coli, pretreatment with a mAb against TNF abrogated development of metabolic acidosis, hypotension, neutropenia, thrombocytopenia, and

microvascular damage.[152,153] Pulmonary hypertension and thromboxane B_2 production was not affected by TNF mAb.

In mice with P. aeruginosa or E. coli bacteremia, prophylactic and therapeutic use of TNF antibody significantly improved survival.[154,155] Administration of TNF mAb, 30 min after LPS infusion, prevented lethality and reduced pro-coagulant responses and morphologic organ damage in adult rhesus monkeys.[156] Baboons survived longer and were less affected clinically when given an anti-TNF mAb 30 min after infusion of live E. coli.[157] Treatment with TNF mAb normalized arterial blood pressure, prevented elevations in serum urea nitrogen and creatinine concentration, and prevented morphologic organ damage in baboons.[157]

Mice given LPS after treatment with high dose TNF mAb were protected from lethality, while mAb against IL-6 failed to afford protection in the same model.[158] Interestingly, when mice were treated with a lower dose of TNF mAb in conjunction with IL-6 mAb, mortality was enhanced.[158] Infusion of IL-6 combined with TNF mAb treatment significantly reduced mortality compared to treatment with low dose TNF mAb alone.[158] These data support the role of TNF in mediating lethal LPS effects, and suggest that IL-6 may play a protective role in the LPS response.

Limited trials have been conducted in clinical cases of sepsis and endotoxemia in human beings.[159,160] Administration of Mu mAb against Hu TNF (CB0006) to human patients with

unresponsive hypotensive shock and presumed sepsis caused an immediate and sustained rise in mean arterial pressures without negative effect.[160] In a multi-center prospective trial, 80 patients with severe sepsis or septic shock were given differing dosage of CB0006 in addition to standard care. While CB0006 treatment did not result in increased survival in the overall study population, a smaller group of patients with circulating TNF activity appeared to benefit from treatment.[160]

Taken as a whole, these studies provide a strong rationale for continuing to develop and test anti-TNF agents for use in clinical cases of endotoxemia. Also promising are strategic combinations such as anti-LPS serotherapy and TNF neutralization, or neutralization of IL-1 and TNF. Effects of these combinations have been shown to be additive in nature and further enhance survival rates when contrasted to sham-treated controls or either treatment alone.[161]

As in other species, considerable evidence supports a role of TNF in the pathogenesis of equine endotoxemia.[20,21,23,24,101] Equine TNF is detected in serum following LPS infusion with maximal activity occurring before most clinical signs and prior to hematologic and metabolic changes.[20,101] Circulating TNF activity was detected in adult horses and foals with naturally occurring diseases associated with endotoxemia, and there was correlation between TNF levels and fatal outcome.[21,23,24,101]

Models of equine endotoxemia have been developed where low dosage of LPS is given that causes significant and repeatable metabolic, hematologic, and clinical responses.[97-110] To help define the role of TNF in these responses, Mu mAb against native Eq TNF were developed and tested in a model of equine endotoxemia. The development, characterization, and testing of these antibodies is described in the following chapters.

CHAPTER 3
ENRICHMENT OF TUMOR NECROSIS FACTOR FROM EQUINE SERUM

Summary

In the horse and other species, tumor necrosis factor alpha (TNF) is hypothesized to be an important proximal mediator of the effects of bacterial endotoxins. As part of an ongoing project to define the role of TNF in equine (Eq) endotoxemia, native Eq TNF was prepared from the serum of horses given lipopolysaccharide (LPS) using immunoaffinity techniques. Antiserum was raised in goats immunized with a peptide corresponding to the N-terminal 15 amino acids of human (Hu) TNF, and was shown to neutralize Eq TNF. Anti-peptide antibodies were purified and used to construct an Eq TNF-affinity column. Horses were injected intravenously (IV) with LPS, blood was collected 90 minutes (min) later, and serum processed from the blood was shown to have high cytotoxic activity for a TNF-sensitive cell line (L929). This serum was loaded onto the Eq TNF-affinity column, eluted in guanidine-HCl, and subjected to anion-exchange chromatography. Approximately 25% of the initial cytotoxic activity was recovered in a final product shown by SDS-PAGE and Western blot to contain a band at 17.6 kDa (Eq TNF). This enrichment scheme resulted in a 14,000-fold enrichment

of TNF specific activity (units (U) TNF activity/mg total protein) from 70 U/mg to 1 X 10^6 U/mg, and produced a reagent suitable for use as an immunogen.

Introduction

Major objectives of research in human beings and horses have been to define the pathophysiological events that occur during endotoxemia, and to find therapies capable of improving clinical outcomes in septic and endotoxemic patients.[2,4,20-24,54,83-117] To be effective clinically, anti-LPS products must cross-react with a diverse group of bacterial endotoxins.[17] In contrast, cytokine-specific agents target mediators produced by patient cells in response to all endotoxins.[2,12] Because a wide array of mediators are produced during endotoxemia, the success of the latter strategy is dependent on identifying and blocking cytokines that are crucial to the LPS response.[2,12] There is convincing evidence that the monokine called tumor necrosis factor alpha (TNF) is a pivotal mediator of LPS effects.[35,42,132-146,149-161] Most importantly, prophylactic and therapeutic use of anti-TNF antibodies has been protective in a variety of experimental models of endotoxemia or sepsis.[149-161]

In horses, circulating TNF activity is present after LPS is given IV.[20,101] Circulating TNF activity has also been found in horses and foals with gastrointestinal disorders

and sepsis.[19-24] Equine TNF produced by LPS-stimulated alveolar macrophages has been partially characterized.[19] Sequential anion-exchange and gel filtration chromatography of stimulated macrophage supernates resulted in a TNF-enriched solution containing about 40% of the original cytotoxic activity, and a specific activity of 4.5 X 10^6 U/mg protein (approximate purification factor of 153).[19] The molecular mass of non-denatured Eq TNF was determined to be 56 kDa.[19]

Previously, antiserums raised in rabbits against synthetic peptides corresponding to specific amino acid sequences of Hu TNF were shown to bind Eq TNF.[162] A peptide corresponding to the N-terminal 15 amino acids of Hu TNF (Hu TNF(1-15)) induced the highest anti-Eq TNF response.[162] In this chapter, the production of anti-peptide antibodies and their application to enrich Eq TNF for use in immunization of mice and monoclonal antibody production is described.

Materials and Methods

Production of equine serum with high cytotoxic activity. Horses were given flunixin meglumine,[a] 1.1 mg/kg bodyweight intravenously (IV). Thirty min after flunixin administration, horses were given 1 - 5 μg/kg endotoxin IV. Endotoxin was bacterial LPS (*E. coli* O55:B5[b]) prepared in

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[a]Banamine, Schering Co, Kenilworth NJ.

[b]Sigma Chemical Co, St Louis, MO.

pyrogen-free water to a concentration of 1 mg/ml, and stored in aliquots at -20 C. Prior to use, an aliquot of LPS was thawed and diluted into 10 ml phosphate-buffered saline solution (PBSS). Whole blood (4 - 8 L) was collected via jugular catheter into citrate anti-coagulant[c] during a 30-min period beginning 75 min after LPS administration. Blood was recalcified by addition of 1 M calcium chloride (final concentration: 15.7 mM), then allowed to clot for 60-min at 37 C. Serum was harvested, clarified by centrifugation (200 X g, 4 C, 15 min), adjusted to 0.02% sodium azide (NaN_3), and stored less then 1 week at 4 C until purification.

Production of caprine antiserum against Hu TNF(1-15). The Hu TNF(1-15) peptide was synthesized using solid-phase methods, modified by addition of tyrosine residue to the carboxy-terminus, and conjugated to bovine thyroglobulin by tyrosine coupling with bis-diazotized benzidine.[163] Conjugated Hu TNF(1-15) was mixed 50:50 with a monophosphoryl lipid A/synthetic trehalose dicorynomycolate/ cell wall skeleton adjuvant[d] (immunizations 1 - 8), or incomplete Freund's adjuvant[a] (immunizations 9 and 10), and used to inoculate 2 goats intramuscularly according to the following schedule: 0.5 mg initially, 1 mg 3 weeks later, followed by 8, 1 - 3 mg boosts at monthly intervals. Serum

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[c]ACD Formula A, Baxter, Deerfield, IL.

[d]Ribi Adjuvant System, Ribi Immunochem Res Inc, Hamilton, MT.

anti-peptide and anti-Eq TNF titers were determined on blood collected 7 - 10 days after each inoculation. Seven to ten days after the 9^{th} and 10^{th} immunizations, 500 ml serum was harvested from blood collected from each goat. This hyperimmune serum was either kept at 4 C for purification in less then 1 week, or stored at -80 C until purification.

Purification of caprine anti-peptide antibodies. A 10-ml anti-peptide-affinity column was constructed by coupling Hu TNF(1-15) to cyanogen bromide (CnBr)-activated agarose.[164] Peptide solution, 5 mg in 5 ml coupling buffer (0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3), was added to 3 g of acid-swollen agarose[e] and mixed gently for 24 h at 4 C. Non-reacted sites were blocked with 20 ml 0.2 M glycine (in coupling buffer) for 26 h at 4 C. The gel was washed 10X, alternating between coupling buffer and 0.1 M acetate buffer, pH 4, then the column was poured. The column was stored between uses in coupling buffer/0.02% NaN_3 at 4 C.

Prior to purification, hyperimmune serum was centrifuged (10,000 X g, 4 C, 20 min), and EDTA was added to a final concentration of 10 mM. All purification steps were performed at 4 C. Serum was loaded onto the column by peristaltic pump at a rate of 1 ml/min. The column was washed with 200 ml coupling buffer, and anti-peptide antibodies were eluted in 20 ml 0.1 M glycine, pH 2.5, followed by 20 ml 100 mM Tris-HCl, pH 8.8. The 40-ml

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[e]CnBr-activated Sepharose 4B, Pharmacia, Uppsala, Sweden.

elution fraction was collected into a tube containing 10 ml 1 M Tris-HCl, pH 8.8, then immediately dialyzed against PBSS. Samples were collected at all stages during the affinity-purification process and tested for anti-peptide and anti-Eq TNF activity.

Construction of an Eq TNF immunoaffinity column. Two batches of purified anti-peptide antibodies (total volume 100 ml) were pooled and concentrated by vacuum-dialysis to 5 ml (36 mg/ml total protein concentration). The antibodies were coupled to 5.7 g of CnBr-activated agarose beads[e] and used to pour a 20-ml affinity column with an Eq TNF-binding capacity of approximately 500 μg. The procedures were identical to those used for making the anti-peptide-affinity column, except that the coupling reaction was carried out at room temperature for 30 min. A "pre-column" was made using pre-immune goat antibody (purified by caprylic acid/ammonium sulfate precipitation[165]) for the purpose of minimizing non-specific binding to the Eq TNF-affinity column.

Immunoaffinity purification of Eq TNF. Two liters of TNF-rich Eq serum were centrifuged (15,000 X g, 4 C, 10 min), filtered[f], and adjusted to 10 mM EDTA. In a 4 C compartment, serum was loaded by gravity onto the 2 columns, in serial (precolumn followed by TNF-affinity column), at a rate of 1 ml/min. The columns were separated, washed with

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[f]Whatman #1, Whatman International LTD, Maidstone, England.

200 - 300 ml 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3, and eluted with 50 ml 4 M guanidine-HCl[a]/2 M NaCl. A single 30-ml fraction corresponding to peak absorbance of the Eq TNF-affinity column eluate at 280 nm was collected and immediately dialyzed against PBSS. Eluate and aliquots collected throughout the affinity-purification process were assayed for TNF and protein content.

Anion-exchange chromatography. The affinity-purified eluate was subjected to anion-exchange chromatography by modification of a technique described previously.[19] All column buffers were adjusted to 0.02% (w/v) octyl glucoside (OG)[a], filtered (0.22 μm) and de-gassed. Sample was dialyzed against 100 mM Tris-HCl, pH 7.8, filtered (0.22 μm), adjusted to 1% OG, and loaded, 1 ml/min, onto a fast-protein liquid anion-exchange column.[g] The column was equilibrated with 100 ml 100 mM Tris, pH 7.8, then eluted with a 160 ml linear 100 - 500 mM Tris-HCl gradient, 4 ml/min. The eluate was monitored for absorbance at 280 nm and 2-ml fractions were collected for immediate TNF assay.

Western blot of Eq TNF. Equine TNF was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), then Western blotting.[166,167] For SDS-PAGE, affinity-purified Eq TNF (AP Eq TNF), anion-exchange-enriched-AP Eq TNF, and recombinant-derived human TNF alpha (rHu TNF), were prepared

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[g]Mono-Q HR 10/10, Pharmacia LKB Biotech Inc, Piscataway, NJ.

by addition of equal volume of 0.1 M Tris-HCl/0.002% bromphenol blue/16% glycerol/3% SDS, in the presence or absence of 20% β-mercaptoethanol, then boiled for 3 min. Samples were loaded (10 μl/lane) onto a 4% polyacrylamide stacking gel, then electrophoresed through a 11% - 22% polyacrylamide gradient separating gel (1.5 mm thick). After completion of SDS-PAGE, samples were transferred electrophoretically to nitrocellulose paper, and the blot was incubated overnight at 4 C, in blocking buffer (10 mM Tris-HCl/200 mM NaCl/0.02% NaN_3/5% non-fat dry milk). For TNF detection, caprine anti-Hu TNF(1-15) antiserum was added, followed by alkaline phosphatase-labeled rabbit anti-goat IgG[h], at 1/100 and 1/1000 dilution, respectively, in blocking buffer, 1 h at room temperature. The blot was rinsed well between incubations in 10 mM Tris-HCl/200 mM NaCl/0.02% NaN_3/0.05% Tween 20. Substrate (NBT, 0.1 mg/ml, BCIP 0.1 mg/ml in 100 mM Tris-HCl/0.1 mM $MgCl_2$/0.02% NaN_3, pH 8.8) was added and the blot was incubated at room temperature until bands were clearly visible (30 min). Molecular masses of immunoreactive Eq TNF and Hu TNF were determined by relative mobility comparison with commercially available molecular weight markers[i], which had been electrophoresed and transferred concurrently.

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[h]H + L, Pierce, Rockford, IL.

[i]Rainbow, Amersham Canada Ltd, Oakville, Ontario.

Assays for TNF activity. Plasma TNF activity of samples was measured in duplicate as cytolytic activity for actinomycin D-primed murine L929 fibroblastoid cells.[170] Cells were seeded into wells of microtitration plates, 3 X 10^4 cells/well, in 0.1 ml media, and allowed to form confluent monolayers for 22 h, 37 C, in 5% CO_2 in air. Serial 2-fold dilutions of duplicate samples were made across the plate, then all wells were adjusted to 5 μg actinomycin D[a]/ml (by addition of 0.1 ml actinomycin D, 10 μg/ml). Plates were incubated (37 C, 20 h, 5% CO_2), then media and dead cells were flicked out of the wells, and remaining adherent (viable) cells were stained with 0.1% crystal violet in a 2% solution of methanol. Cell survival was quantified by measuring optical density (OD) at 540 nm using an automated microtitration plate reader and software.[j] On each plate, a column of negative control wells incubated in medium alone (100% survival), and a column of positive control wells incubated in 1/100 dilution of Eq TNF reference serum (100% cytotoxicity) were included. The mean OD for the positive control wells was subtracted from the OD of all other wells. Sample Eq TNF activity was the inverse of the dilution causing exactly 50% cytotoxicity. This point was determined by interpolation between dilutions causing OD just above and just below OD at

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[j]Titertek Multiskan MC, Titersoft II, Flow Laboratories Ltd, Irvine, Scotland.

50% cytotoxicity. By definition, the titer that results in 50% cytotoxicity contains 1 U TNF activity/ml.

A dilution of freshly-thawed Eq serum, (shown by multiple assays to contain 7,500 U of TNF activity/ml serum), was included at the beginning and end of each assay. The mean titer of this serum was normalized to 7,500 U, and that factor was applied to all samples to normalize results between assays.

Assay for anti-TNF activity. Equine TNF-binding activity was determined by cytotoxicity-reduction assay. Thirty microliter amounts of serial ten-fold sample dilutions were each mixed with 270 μl Eq TNF reference serum (1/35) and 200 μl of well-washed, 10% (w/v) Staphylococcus aureus in PBSS.[k] Following incubation at 4 C for 4 h, samples were centrifuged (15,000 X g, 5 min) and supernates were assayed in duplicate for residual cytotoxicity. By comparison to PBSS control, the sample dilution that resulted in 50% reduction in reference serum cytotoxicity was determined by interpolation. Equine TNF-binding activity was calculated as the inverse of the sample dilution causing 50% reduction in reference serum cytotoxicity, multiplied by half the reference serum TNF activity.

Assay for anti-peptide activity. An ELISA was developed to measure caprine immune serum Hu TNF(1-15)

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[k]IgG sorb, The Enzyme Center Inc, Malden, MA.

binding activity. Unless otherwise stated, volumes added were 0.1 ml/well, incubation temperature was 37 C, and incubation time was 2 h. For all washes, wells were filled (0.45 ml) with PBSS/0.05% Tween 20, then the solution was flicked out, and the plate tapped on a paper towel. The Hu TNF(1-15) peptide, 2 μg/ml in coating buffer (0.06M carbonate buffer, pH 9.6), was added to all wells of a standard ELISA plate,[1] with the exception of the second column of wells used as a positive control. In the positive control column, purified pre-immune goat IgG, 2 μg/ml in coating buffer, was added. Plates were incubated at 4 C overnight, then washed twice. All wells were blocked with 0.4 ml 1% BSA/0.02% NaN_3, incubated, washed twice, and PBSS was added for dilutions. Neonatal calf serum[a], 1/25 in blocking buffer, was added to all wells of column 1 as a negative control, and PBSS was added to positive control wells. Pre-immune and test immune serums, 1/25 in blocking buffer, were added to assay wells and assayed in duplicate through 12 2-fold dilutions. The plates were then incubated, washed 4X, and alkaline phosphatase-labeled rabbit anti-goat IgG was added to all wells. Finally, plates were incubated 1 h, washed 4X, and substrate solution (1 mg/ml p-nitrophenyl phosphate disodium in 10% diethanolamine/0.5 mM $MgCl_2$), was added to all wells (50 μl/well), and plates were developed for 30 min. At this point, the reaction was

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[1]Nunc Maxisorp, Marsh Biomedicals, Rochester, NY.

stopped with 0.1 M EDTA, 50 μl/well, and OD at 405 nm was measured using an automated microtitration plate reader and software.[j] Titers were calculated as the reciprocal of the dilution that resulted in an absorbance reading of 25% of the average absorbance of the positive control wells.

Protein determinations. All protein determinations were made by the bicinchoninic acid method using commercially available reagents.[m]

Results

Production and purification of anti-peptide antibodies. No anti-peptide or anti-Eq TNF activity was present in caprine serum collected prior to immunization (figure 3-1). After 2 immunizations, Eq TNF-binding titers were present in both goats (1,391 U/ml and 1,708 U/ml). After 6 immunizations, maximum titers were present (60,000 and 40,000 U/ml, respectively). Peptide-binding activity was also present after the first 2 and all subsequent immunizations (figure 3-2).

All anti-Eq TNF activity was removed from immune serum during a single passage over the anti-peptide column (figure 3-3). Surprisingly, greater then 100% of the loaded anti-TNF activity was recovered in the elution fraction. If a small amount of fall-through was added to the anti-peptide column eluate, a reduction in anti-Eq TNF activity occurred

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[m]Pierce BCA protein assay reagent, Pierce, Rockford, IL.

as compared to addition of equal volume PBSS. The affinity-purified antibody solution had protein concentration of 1.8 mg/ml, and TNF-binding activity of 5 X 10^5 U/ml.

Imunoaffinity purification of Eq TNF. Serum collected from horses given LPS had 3000 - 5000 U/ml TNF activity. This activity was unchanged after the pre-column, but was completely depleted by a single passage over the Eq TNF-affinity column. During guanidine-HCl elution of the Eq TNF-affinity column, a single 30-ml fraction corresponding to peak absorbance at 280 nm was collected that contained approximately 2 X 10^5 U/ml TNF activity, and 2 mg/ml total protein concentration (table 3-1).

Anion-exchange enrichment of affinity-purified Eq TNF. Anion-exchange chromatography was successful in further enriching affinity-purified Eq TNF by separation from other protein contaminants (figure 3-4). Table 3-1 summarizes Eq TNF sample characteristics during enrichment.

Western blot analysis of Eq TNF. Supportive evidence for identity of the recovered cytotoxic activity as Eq TNF was obtained by Western blot analysis in which a distinct band developed at 17.6 kDa and ran closely with a band of rHu TNF (figure 3-5). This band was not changed by reduction, however a second major band running at the top of the gel was reduced. By comparison of relative mobilities, Eq TNF appears to be slightly heavier than rHu TNF (17.6 kd vs 17.0 kd).

Discussion

The goal of this investigation was to produce enriched Eq TNF in sufficient quantities for immunization of mice and production of monoclonal antibodies against Eq TNF. Because anti-Eq TNF antibodies have potential clinical application, active, native Eq TNF was felt to be the immunogen of choice to produce anti-TNF reagents with excellent in vivo neutralizing activity. Recombinant technology is commonly used to produce large quantities of protein; however, bacterially-expressed proteins do not have potentially important post-translational modifications such as glycosylation, and this can affect protein immunogenicity.[169]

Immunoaffinity techniques have been used to isolate proteins from complex mixtures such as serum.[170] The success of this technique depends on identifying an appropriate affinity ligand to which the molecule of choice will selectively bind to, and can be separated from, without loss of activity.[170] Two observations were exploited to produce a suitable affinity ligand for isolating native Eq TNF. First, it had been ascertained that antibodies raised against a synthetic peptide corresponding to the N-terminus of Hu TNF neutralized Hu TNF activity.[163] Second, it was shown that these anti-peptide antibodies cross-reacted with native Eq TNF.[162] The Hu TNF (1-15) peptide was synthesized in large quantities and used to produce and purify caprine anti-peptide antibodies, which were subsequently used as an

affinity ligand for Eq TNF. The relatively exposed position, linear configuration, and highly conserved sequence of the N-terminus of TNF may explain why this strategy was successful in producing anti-peptide antibodies that cross-reacted with Hu TNF and Eq TNF.[129] It was interesting to observe that greater then 100% of the anti-Eq TNF activity loaded onto the anti-peptide-affinity column was recovered upon elution. This indicates that some factor was present in the serum that interfered with the interaction between anti-peptide antibodies and Eq TNF. Anion-exchange chromatography was successful in further enriching the affinity-purified Eq TNF as previously described.[19]

Western blot analysis revealed that serum Eq TNF (17.6 kDa) had a slightly higher molecular weight then that observed by us for rHu TNF (17 kDa) or that observed by others for recombinant-derived Eq TNF (17 kDa).[22,123] This may indicate that native Eq TNF is glycosylated. Murine TNF has been shown to be a glycoprotein but Hu TNF is not.[123,171] The molecular weight of denatured Eq TNF is about 1/3 that reported for non-denatured Eq TNF (approximately 56 kDa), and correlates well with an active trimeric form of Eq TNF, as reported for other species.[19] Another major band of protein identified by Western blot ran at the top of the gel, and reduced to two bands, one which was > 46 kDa and another which was between 21.5 kDa and 30 kDa. Immunoglobulin would behave in this fashion, and this band

could be anti-peptide antibody which leached off of the Eq TNF-affinity column and was detected by the alkaline phosphatase-labeled anti-caprine immunoglobulin reagent.

The affinity/anion-exchange techniques were easy to perform, and a source of Eq TNF is available in the form of serum processed from blood collected after horses are given LPS. This enrichment strategy resulted in a 14,000-fold enrichment of TNF specific activity from 60 U/mg to 1 X 10^6 U/mg, and produced a reagent suitable for immunizing mice.

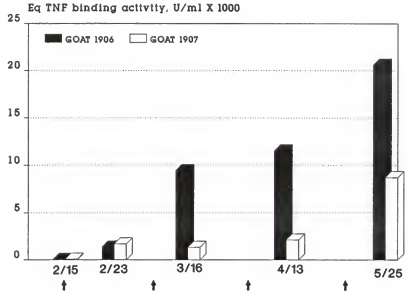

Figure 3-1. Anti-Eq TNF activity of serum from goats immunized with a peptide corresponding to the N-terminal 15 amino acids of Hu TNF. Arrows denote inoculation dates.

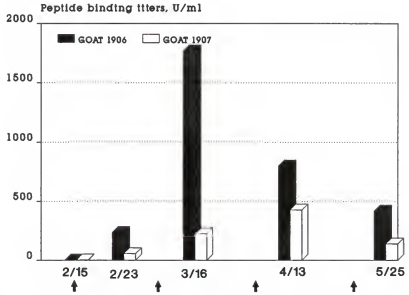

Figure 3-2. Anti-peptide activity of serum from goats immunized with a peptide corresponding to the N-terminal amino acids of Hu TNF. Arrows denote inoculation dates.

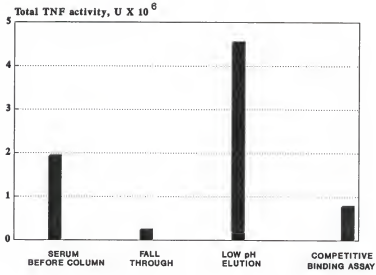

Figure 3-3. Total anti-TNF activity of goat anti-peptide antibodies during affinity purification. The first bar denotes loaded activity, the second bar activity that fell through the column, and the third bar total anti-TNF activity eluted off the column. Note that this is > 100% of loaded activity. Addition of fall through to eluate caused reduced anti-TNF activity, compared to addition of equal volume PBSS (fourth bar). This indicates that a serum factor interferes with interaction of anti-peptide antibody and Eq TNF.

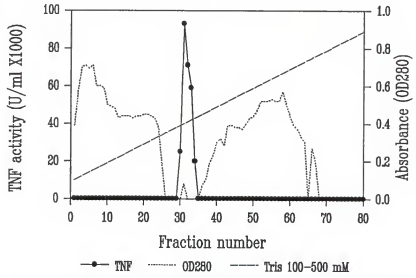

Figure 3-4. Anion-exchange chromatography of Eq TNF. Buffers contained 0.02% octyl glucoside (OG). Sample (20 ml affinity-purified Eq TNF) was adjusted to 1% OG, loaded over a 10 cm X 10 cm Mono-Q FPLC column, equilibrated with 100 mM Tris-HCl, pH 7.8, and eluted with 160-ml linear 100-500 mM Tris-HCl gradient. Eluate was monitored at 280 nm and 2-ml fractions were collected and assayed immediately for TNF activity.

Table 3-1. Enrichment steps for equine TNF

FORM	Volume (ml)	Protein (mg/ml)	Sp. Act. (U/mg)	TNF[a] (μg)	Recovery (%)
serum	2000	70.0	70	100	---
IAP	25	3.0	7×10^4	50	50
ANEX	25	0.1	1×10^6	25	25

[a]TNF amount in μg is calculated using the specific activity of rHu TNF in bioassay (1×10^8 U/mg)
IAP = immunoaffinity-purified Eq TNF
ANEX = anion-exchange-enriched, IAP Eq TNF

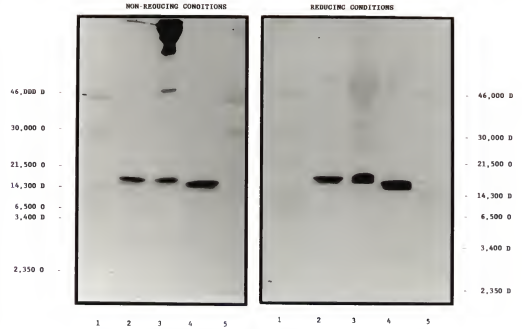

Figure 3-5. Western blot analysis of Eq TNF. Lanes 1,5 = molecular weight markers. Lane 2 = anion-exchange-enriched Eq TNF, lane 3 = affinity-purified Eq TNF, lane 4 = rHu TNF. Samples were subjected to SDS-PAGE on 11-22% gradient gel, then electrophoretically transferred to nitrocellulose paper. Tumor necrosis factor was detected by addition of 1/100 goat anti-Hu TNF(1-15) and 1/1000 alkaline phosphatase-labeled rabbit anti-goat IgG and substrate.

CHAPTER 4
PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST EQUINE TUMOR NECROSIS FACTOR

Summary

Monoclonal antibodies (mAb) were produced against equine (Eq) tumor necrosis factor alpha (TNF) prepared from the serum of horses given endotoxin. Four mAb selected for high anti-Eq TNF activity were isotyped (three IgG_1 and one IgG_{2a}) and produced as ascites in pristane-primed BALB/c mice. Binding and neutralizing activities of the mAb (as ascites) for Eq TNF were determined by cytotoxicity reduction in a standard TNF bioassay with (binding) or without (neutralizing) immobilization of antibody-TNF complexes on a suspension of heat-killed Staphylococcus aureus. Activity of mAb for Eq TNF ranged from 5.1×10^4 units (U)/ml to 2.9×10^6 U/ml (binding) and 5.5×10^3 U/ml to 2.0×10^6 U/ml (neutralizing). When assayed for activity against human, murine, and rabbit TNF, 1 of 4 mAb had detectable activity against rabbit TNF. No other cross reactivity was detected. Computer-generated analysis of the antigenic index of Eq TNF was similar to published structural characteristics of TNF from other species. Two mAb were epitope-mapped by peptide binding analysis to

sequences within areas that were predicted to be highly antigenic (residues 63 - 79 and 109 - 118, respectively). The other two mAb failed to recognize any Eq TNF peptide sequence, probably indicating discontinuous or longer epitope specificities.

Introduction

Over the past decade, TNF has emerged as an central participant in mammalian inflammatory and immune responses.[18,41,42,172] Tumor necrosis factor alpha regulates growth, differentiation, and function of hematopoietic, immune, and other cells.[41,173] In addition to anti-tumor effects, TNF enhances immunity to bacterial, viral, and parasitic agents.[174,175,176] In excessive amounts, TNF mediates endotoxemia[12,18,33,35,134], cachexia of chronic disease[18,177], and is implicated in autoimmune disorders.[178,179] High circulating TNF activity has been associated with death in human beings[77,145] and horses[21,23,24] with naturally occurring sepsis or endotoxin-associated diseases. Antibodies against TNF have been protective in a variety of models of endotoxemia.[149-161]

Human[123,124], mouse[122], rat[180], rabbit[125], cat[181], pig[126], cow[127], and horse[22] TNF have been molecularly cloned and produced as recombinant proteins. Bioactive TNF exists as a 50 kDa trimer of identical β-pleated subunits.[129] Tumor necrosis factor alpha is secreted mainly by macrophages in

response to a variety of stimuli such as lipopolysaccharide[1,8], phorbol esters[182], and cytokines.[174,182] Secreted TNF is bound rapidly to specific high-affinity cell-surface receptors distributed throughout most tissues and exerts pleiotropic effects through a diversity of post-receptor signalling mechanisms.[39,130] Circulating soluble TNF receptors (sTNFR), also called TNF binding proteins, are naturally occurring inhibitors of TNF activity.[183,184] Elevated sTNFR levels have been demonstrated in humans in response to endotoxin challenge and in patients with sepsis.[185,186] Preliminary evidence indicates that increases in sTNFR may be due to shedding of cell-surface receptors.[187] This provides a dual mechanism for protection, initially by transiently desensitizing cells to TNF and, additionally, by providing increased sTNFR to neutralize circulating TNF activity.

Anti-TNF antibodies have been used to study structural and functional aspects of TNF[163,188,189], to develop immunoassays for measurement of TNF concentration in biological fluids[22,190-192], and to define the role of TNF in septic shock and endotoxemia.[131,146,149-161] Monoclonal antibodies were developed previously against recombinant-derived Eq TNF (rEq TNF), and were used to develop an Eq TNF immunoassay.[22] In this chapter the production and characterization of monoclonal antibodies against serum Eq TNF is described.

Materials and Methods

Monoclonal antibody production. Tumor necrosis factor was prepared from the serum of horses given endotoxin using affinity techniques and anion-exchange chromatography. Detailed procedures are described in chapter 3. All mouse immunizations, hybridoma manipulation, and ascites production were performed under contract by the University of Florida ICBR Hybridoma Facility, Gainesville, Florida. Enriched Eq TNF containing approximately 1 X 10^6 U Eq TNF was mixed 50:50 with adjuvant[a] and used to inoculate a BALB/c mouse subcutaneously. The same amount was given 3 and 6 weeks later. Blood was collected 10 days after 2 and 3 inoculations and assayed for anti-Eq TNF activity. After 3 inoculations, anti-Eq TNF binding activity was detected (3,165 U/ml serum). Eight weeks after the third inoculation, the mouse was given 3.5 X 10^6 Eq TNF U in 0.75 ml phosphate-buffered saline solution (PBSS) by intraperitoneal injection. Fusion was performed 3 days later.

Splenocytes from the immunized mouse were mixed with murine myeloma Sp2/0-Ag14 cells (7:1 ratio of spleen to myeloma cells) and fused with polyethylene glycol as described previously.[193] Hybridoma cultures were selected for expansion and cloning based on ability of culture supernates to reduce Eq TNF cytotoxicity in standard bioassay. Five

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[a]RIBI MPL + TDM Emulsion, Ribi Immunochem Res Inc, Hamilton, MT.

positive hybridomas were cloned by a modified limiting dilution technique over fibroblast feeder cell cultures and single-colony wells were selected microscopically.[194] The 4 clones with the highest binding and neutralizing activities for Eq TNF (HL 801, 806, 819, 823) were selected for production as ascites in pristane-primed BALB/c mice.[195] In addition, supernates from multiple unrelated hybridomas were screened for anti-TNF activity. The mAb selected for control purposes (HL262) was produced against a Xenopus oocyte nuclear protein and possessed no detectable anti-Eq TNF activity.

Isotype determinations. Isotype determinations were made by ELISA[b] or with quick typing sticks using mouse-isotype specific antibodies.[c]

Sources of TNF. Blood was collected from a horse 90 min after LPS was given intravenously (IV). Serum was separated and stored in aliquots at -80 C (chapter 3). Reference serum contained 7,500 U/ml of Eq TNF activity and was diluted and used both for determining anti-Eq TNF activity and as a positive control in bioassays. Native rabbit TNF in the form of serum containing 25,000 U/ml was

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[b]Immunoselect, GIBCO Laboratories, Grand Island, NY.

[c]Amersham, Arlington Heights, IL.

kindly provided as a gift.[d] Purified recombinant-derived human and murine TNF-α were purchased.[e]

Assays for anti-Eq TNF activity. For hybridoma screening, duplicate 100 μl amounts of undiluted culture supernates or medium (controls) were mixed with 100 μl diluted Eq TNF (final activity: 75 U/ml) and 200 μl 10% (w/v) suspension of washed, killed Staphylococcus aureus.[f] Samples were incubated, 4 C, 4 hours (h), then TNF-antibody-S. aureus complexes were removed from solution by centrifugation (15,000 X g, 10 min). Residual cytotoxicity of each sample supernate was determined by bioassay over L929 cells as described in chapter 3. Hybridoma cultures were selected for expansion and cloning if culture supernates reduced control TNF titer $\geq$ 40%.

For supernates of cloned hybridomas, initial screening was identical to that described above. Positive clones were selected, expanded, and subjected to secondary screening in which serial 5-fold dilutions of clone supernates, starting at 1/25 dilution, were assayed for binding activity. Exact antibody dilution that reduced the TNF titer by 50% (i.e. to 37.5 U/ml) was determined by interpolation, and the reciprocal of this calculated dilution X 37.5 was the binding titer for Eq TNF.

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[d]Dr. George Gifford, Univ of Florida, Gainesville, FL.

[e]Genzyme, Boston MA.

[f]IgGsorb, The Enzyme Center Inc, Malden MA.

For assays comparing neutralizing and binding activities of ascites for Eq TNF, serial 2-fold dilutions of ascites were made, starting with dilutions of 1/20 (HL 819, 823, 262), 1/200 (HL 806), or 1/2000 (HL 801). Assays were performed as for hybridoma screening except that medium was added instead of S. aureus for neutralizing assays. Binding and neutralizing activity for Eq TNF was calculated by multiplying the amount of TNF bound or neutralized (control TNF titer minus sample TNF titer) by the antibody dilution. Activity generated at each dilution was averaged and reported as the mean ± SEM U of Eq TNF bound or neutralized per ml ascites (U/ml).

Assays for activity against TNF from other species. A modification of the neutralizing assay described above was used to determine the cross-reactivity of anti-Eq TNF mAb with TNF of other species. For this assay, media was flicked off confluent L929 cell monolayers contained in wells of microtitration plates. Duplicate serial 2-fold dilutions of antibody were added to wells followed by addition of equal volume TNF, diluted to the following activities; rabbit, 2.75 U/ml, Eq, 53.9 U/ml, murine, 4.1 U/ml, and human, 13.3 U/ml. Neutralizing activities were calculated by identifying the antibody dilution that reduced TNF activity to 1 U/ml (50% cytotoxicity as determined by interpolation between adjacent dilutions), then multiplying that dilution by the U/ml TNF added to the well minus the 1

U/ml TNF activity remaining in the well at the 50% cytotoxicity point.

Antigenic index analysis of Eq TNF. An antigenic index plot compiled from analysis of molecular flexibility, hydrophilicity, and surface accessibility, was generated from the primary Eq TNF[196], sequence using a commercially available computer software program.[g]

Specific peptide binding analysis. Binding analyses of HL 801, 806, 819, and 823 to a series of decapeptides corresponding to specific amino acid sequences within Eq TNF,[196] were performed using a commercially available simultaneous solid-phase peptide synthesis and immunoprobing kit and software.[h] A synthesis schedule was generated with the computer software to produce 74 Eq TNF decapeptides, each offset by 2 amino acids. Specified reactive Fmoc-amino acid esters were coupled to reactive amino groups of a derivatized cellulose membrane, then residual groups were blocked by acetylation. Removal of Fmoc-groups was performed to generate new reactive amino groups, and the cycle repeated until all peptides were synthesized. After the final cycle, peptides were N-terminally acetylated and side chain deprotected for subsequent immunoprobing.

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[g]Surfaceplot, v 1.10 1986, Synthetic Peptides Inc, Richmond Hill, CA.

[h]SPOTs, Cambridge Research Biochem Ltd, Wilmington, DE.

Unless otherwise noted, all immunoprobing steps were done at room temperature and wash steps were performed 3X by addition of 20 ml Tris-buffered saline solution, pH 8.0 (TBSS)/0.05% Tween-20 and rocking for 10 min. Prior to epitope analysis, the membrane with completed peptides was washed with TBSS then blocked overnight in 20 ml blocking buffer (membrane blocking buffer/TBSS/5% sucrose). The following morning, the membrane was washed, then the mAb of interest as ascites (100 μl of HL 801 and 806, 200 μl of HL 819, or 400 μl of HL 823) in 20 ml blocking buffer was added for 3.5 h. The membrane was washed and β-galactosidase-conjugated rabbit-anti mouse IgG[i], diluted 1:200 in blocking buffer, was added for 2 h. The membrane was washed twice, then twice more with PBSS. Signal development solution (0.048% 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside/1 μM $MgCl_2$/0.1% potassium ferricyanide/PBSS, 10 ml) was added and color development monitored until positive reactions could be distinguished clearly. Probes were considered negative if strong color development was not seen within 60 min. The developed membrane was washed twice with PBSS, then peptides were regenerated for the next probe. A single monoclonal antibody was used for each probe of the membrane in the order of HL801 first, HL806 second, HL819 third and HL823 last.

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[i]Cambridge Research Biochem Ltd, Wilmington, DE.

Results

Monoclonal antibody production and characterization. During the initial hybridoma screening, 378 culture supernatants were assayed and 74 were kept for expansion and re-screening. From the secondary screen, 14 cultures were identified as positive and saved. Of these cultures, 4 were strongly positive (reduced Eq TNF titer by 100%), 2 were moderately positive (reduced control Eq TNF activity by 64% to 82%) and the rest were weakly positive (reduced control Eq TNF activity by 40% to 55%).

For each of 5 cloning procedures, 100 supernates were screened initially and 80% - 90% of these reduced TNF activity by at least 80%. Clones from each of the original positive cultures were selected, expanded, and designated HL 801, 806, 819, 823, and 826. The four mAb with the highest anti-Eq TNF activity (HL 801, HL806, HL819, HL823) were produced as ascites and characterized further.

As shown in tables 4-1 and 4-2, all 4 anti-Eq TNF ascites had high, specific activity against Eq TNF. Ascites from HL 801 and 806 had similar Eq TNF binding and neutralizing activities, while that from HL 819 and 823 had approximately 10-fold higher binding vs neutralizing abilities. One mAb, HL 819, cross-reacted with rabbit TNF. None of the other mAb neutralized TNF from other species.

Antigenic index analysis of Eq TNF. The antigenic index plot predicted rrom the primary Eq TNF sequence is

depicted in figure 1. Residues from 2 - 8, 20 - 23, 65 - 68, 100 - 114, 126 - 133, and 138 - 140 had the highest antigenic scores.

Specific peptide binding analysis. The first probe, using ascites containing HL 801, developed two overlapping peptides corresponding to Eq TNF amino acid sequences 59 - 68 and 63 - 72. Ascites containing HL 819 developed a single peptide corresponding to Eq TNF sequence 109 - 118. In general, once a peptide had developed it would tend to develop slightly during each subsequent probing with other antibodies. Very slight variations in color development of some peptides were seen with all probes but this was not interpreted as specific peptide recognition.

Discussion

Monoclonal antibodies produced against serum Eq TNF exhibited high-titered anti-Eq TNF activity. In agreement with previous reports on anti-TNF characteristics, these mAb showed little cross-reactivity with TNF from other species.[197-199] Previously, monclonal antibodies against rEq TNF were reported to have no activity against Eq lymphotoxin, native or recombinant-derived murine TNF, or recombinant-derived porcine TNF.[22] These anti-rEq TNF mAb differed from our anti-serum Eq TNF mAb in that they exhibited some cross-reactivity with recombinant-derived human TNF, whereas ours did not. Because TNF protein

sequence appears to be highly conserved among species (70% to 80% homology between equine, human, rabbit, and murine TNF), it may seem surprising that anti-TNF antibodies exhibit little species cross-reactivity. Structural studies of TNF trimer may provide some explanation for this observation. Sequences which are most highly conserved amongst species generally have low antigenic index, while regions with sequence variations are found in the antibody-accessible surfaces of the TNF trimer.[129,188] Interestingly, in contrast to anti-TNF activity of antibodies, TNF activity itself is generally not species specific.[200]

The main-chain topography of a TNF monomer and TNF amino acid sequence alignment[201] are illustrated in figures 4-2a and 4-2b, respectively, for reference purposes. Antisera raised in rabbits and mice against predicted antibody-accessible peptide sequences of human TNF were used previously to define further immunogenic regions of the TNF molecule.[163,188] These studies identified antigenic sequences in the TNF trimer associated with the N-terminus (1-15, 1-31)[163], and (1-23)[188], and the connecting loops between β-strands of each TNF monomer (65-79, 98-111, 124-141)[163], and (35-37, 95 - 116, 117 - 136).[188] Regions associated with the β-strands or the carboxy-terminus were poorly antigenic. Our analysis of the primary sequence of Eq TNF predicted a similar pattern of antigenicity, and peptide-binding analysis identified epitope specificities within these

regions for 2 of 4 mAb against Eq TNF. One mAb, HL801, bound decapeptides 59-68 and 63-72, indicating recognition of the sequence shared by these 2 peptides (63-68). We have no explanation (other than technical error) for the observation that HL801 did not bind peptide 61-76. HL819 showed strong binding to peptide 109-118 and HL 806 and 823 did not bind to any of the synthetic peptides. Since the same peptides were probed and regenerated sequentially, it is important to note that positive results for HL819 were obtained after two probes were performed. This demonstrates that the peptide membrane was generally unaltered by immunoprobing. However, peptides bound by HL801 and HL819 continued to show some development with subsequent immunoprobings, indicating that regeneration of a specific peptide may not have occurred. Although it is likely that HL 806 and 823 bind to discontinuous or longer epitopes on the Eq TNF molecule, the possibility that they bind one of the epitopes identified by HL 801 and 819 cannot be ruled out without further analysis.

Neutralizing anti-TNF antibodies have generally been defined as those that interfere with TNF cytotoxicity for TNF sensitive cell lines in laboratory assays. Immunoreactive TNF concentration detected with neutralizing anti-TNF mAb correlates closely with TNF bioactivity.[22,185,191,192,198,199] Determination of circulating bioactive TNF has prognostic value, but does not reflect

total TNF release because TNF-sTNFR complexes are not measured. An assay system capable of detecting TNF-sTNFR complexes may give additional information reflecting the total extent of TNF participation in a pathological process.

Previous work in mice has shown that antibodies against peptides corresponding to TNF sequences 95 - 116 have little TNF neutralizing activity.[188] This sequence is located towards the top of the TNF trimer and away from proposed receptor binding sites (refer to figures 4-2a and 4-2b). Two of our mAb exhibited much higher binding then neutralizing activities against Eq TNF. One of these, mAb HL816, was mapped to Eq TNF sequence 109 - 118. These antibodies may be suitable for developing an immunoassay for evaluation of total TNF concentration.

In conclusion, mAb developed against serum Eq TNF had little cross-reactivity with human, murine, or rabbit TNF. Two of these mAb had neutralizing capacities for Eq TNF and may be suitable for testing as potential therapeutic agents for use in Eq endotoxemia. Monoclonal anti-Eq TNF antibodies should be useful reagents in further elucidating the role of TNF in equine endotoxemia.

Table 4-1. Binding and neutralizing activities of monoclonal antibodies for equine TNF.

ID	Isotype	Specificity	Binding Titer (U/ml)	Neutralizing Titer (U/ml)
HL262	$IgG_{1,k}$	Oocyte Prot	< 2112	< 1690
HL801	$IgG_{1,k}$	Eq TNF	2,910,000	1,950,000
HL806	$IgG_{1,k}$	Eq TNF	525,000	358,000
HL819	$IgG_{1,k}$	Eq TNF	40,300	1,900
HL823	$IgG_{2a,k}$	Eq TNF	50,910	5,488

Table 4-2. Monoclonal antibody activities for equine, murine, human, and rabbit TNF.

	TNF Neutralizing Activity (U/ml)			
Antibody	Horse	Mouse	Human	Rabbit
HL801	823,200	< 31	< 169	< 11
HL806	22,100	< 31	< 169	< 11
HL819	790	< 31	< 169	510
HL823	983	< 31	< 169	< 11

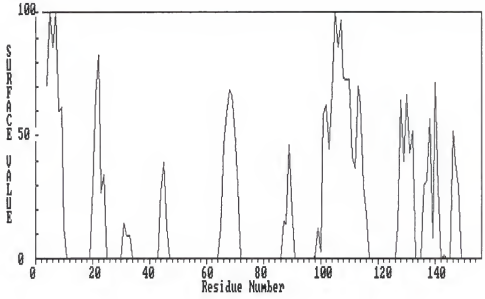

Figure 4-1. Antigenic index analysis of equine TNF. A composite analysis of molecular flexibility, hydrophilicity, and surface accessibility was generated from the primary amino acid sequence of Eq TNF[198], and used to predict antigenicity. Amino acid sequences with scores ≥ 50 were considered highly antigenic (2 - 8, 20 - 23, 65 - 68, 100 - 114, 126 - 132, and 138 - 140). The computer program used was Surfaceplot, v 1.10, 1986, Synthetic Peptides Inc., Richmond Hill, CA.

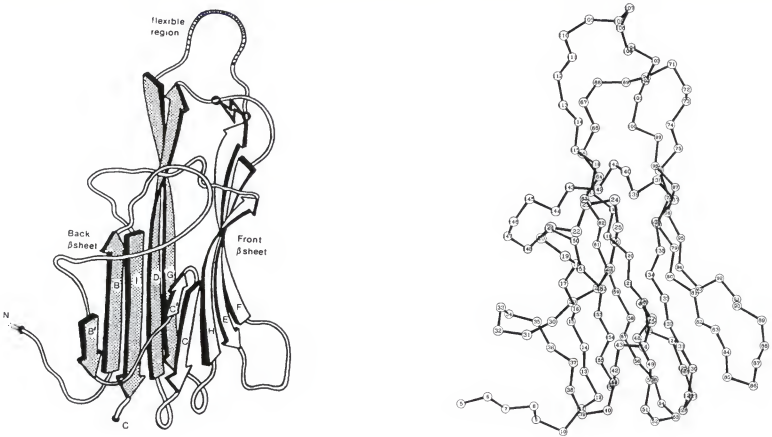

Figure 4-2a and 4-2b. Topographical structure of human TNF (figure 4-2a) and carbon skeleton tracing of the human TNF molecule (figure 4-2b). Reprinted with permission from Marcel Dekker Inc, New York, NY.[201]

CHAPTER 5

EFFECT OF TREATMENT WITH A MONOCLONAL ANTIBODY AGAINST EQUINE TUMOR NECROSIS FACTOR ON CLINICAL AND HEMATOLOGIC RESPONSES OF HORSES GIVEN ENDOTOXIN

Summary

Tumor necrosis factor alpha (TNF) is an important mediator of endotoxin-induced pathophysiology. To help define the role of TNF in equine (Eq) endotoxemia, treatment with a murine monoclonal antibody (mAb) against Eq TNF was evaluated in adult miniature horses given endotoxin. Five horses were given anti-TNF mAb at dosage of 1.86 mg/kg, intravenously (IV), and 5 were given control mAb. Five minutes later, lipopolysaccharide (LPS, E. coli 055:B5), 0.25 μg/kg, was given to all horses by bolus IV infusion. Clinical signs were monitored at intervals up to 24 h after LPS infusion and blood was taken for determination of white blood cell (WBC) count, packed-cell volume (PCV), plasma total protein (TP) concentration, and plasma TNF activity. Reduction of plasma TNF activity in anti-TNF-treated horses was highly significant ($P < 0.001$) compared to control-treated horses. Horses given anti-TNF mAb had significantly ($P < 0.010$) improved clinical abnormality scores, lower heart rates, and higher WBC count, as compared to horses given control mAb. Rectal temperature, respiratory rate,

PCV, and plasma TP concentration were not significantly different between groups. Neutralization of LPS-induced TNF activity reduces the hematological and clinical responses of horses given LPS IV.

Introduction

Endotoxemia has received much attention in equine literature since the 1970s, when it was first observed that horses given LPS exhibited many of the symptoms seen in equine patients suffering from enteric diseases.[5,6,83-117] The extensive equine gut is host to a large gram-negative bacterial flora, and conditions which disrupt the mucosal barrier can result in systemic circulation of endotoxins.[5,9] Some of the enteric disorders that may be associated with endotoxemia in the horse include intestinal obstruction and infarction, duodenitis/proximal jejunitis, salmonellosis, equine monocytic ehrlichiosis, nonsteroidal antiinflammatory drug toxicity, carbohydrate overload, cantharidin toxicity, and peritonitis.[202] Non-enteric disorders can also be complicated by endotoxemia if caused by gram-negative bacteria. Major examples in the horse are neonatal sepsis, pleuropneumonia, and metritis.[203] Endotoxemia has been linked to the development of laminitis in equine carbohydrate overload models.[5] However, the relationship between endotoxemia and laminitis is unclear, since only one study in which horses were given LPS (by hepatic portal infusion)

reported occurrence of mild, acute laminitis.[95] It is obvious that many important equine diseases are associated with endotoxemia, and that understanding the pathogenesis of endotoxemia in horses could lead to improved treatment of these conditions.

The difficulty in effectively treating endotoxemic patients is not surprising considering the rapid cascade of mediators that are released when macrophages and other host cells interact with LPS.[2,4,12-15] Blockade of the LPS-host cell interaction with antibodies directed against core-lipid A epitopes (shared by all gram-negative bacteria) has been attempted clinically with limited success in human beings.[17] In horses, anti-LPS serotherapy also has had mixed results.[99, 110-113], In one instance, anti-core-lipid A antiserum actually worsened the clinical course of foals given LPS.[110] The clinical efficacy of specific mediator blockade during endotoxemia is likewise limited unless the targeted mediator plays a pivotal role in the response to LPS. For instance, treatment with thromboxane synthetase inhibitor or platelet-activating factor receptor antagonist did not attenuate responses of horses given LPS.[98,115] Blocking the formation of products of cyclooxygenase activity by use of flunixin meglumine did abrogate clinical and hemodynamic alterations in horses given LPS, but did not prevent death.[87-93,97,98,100,115]

Recently, TNF has emerged as a pivotal mediator of LPS effects.[118-161] Tumor necrosis factor alpha is released by macrophages early during the course of endotoxemia.[141-148] Circulating TNF activity correlates with the severity and outcome of disease, infusion of TNF produces an endotoxemic shock-like syndrome, and TNF blockade confers significant protection against the effects of LPS administration.[141-161] Circulating TNF activity has been detected in horses and foals given LPS, and in horses and foals suffering from naturally occurring diseases.[19-24] In this chapter, the effect of treatment with a mAb against Eq TNF on the clinical signs, hematological responses, and circulating TNF activity of miniature horses given LPS will be described.

Materials and Methods

Horses. Ten miniature horses, 8 male and 2 female, aged between 9 months and 3 years, and weighing between 75 and 151 kg, were studied. All horses appeared normal on physical examination and had normal WBC count, PCV, plasma TP concentration, and fibrinogen concentration. Three days prior to experiments, horses were transported to the research facility where they were kept on a grass paddock and fed coastal Bermuda hay ad libitum, and 350 g of grain twice daily.

Antibody treatments. An $IgG_{1,k}$ isotype murine mAb (HL801), produced against native Eq TNF, was given to horses

in the treatment group. An isotype-matched mAb (HL262), with no reactivity to Eq TNF, was given to horses in the control group. Both antibodies were described in chapter 4. Antibody treatments were given in 100 ml sterile 0.9% saline solution by IV infusion over 15 min at a dose of 1.86 mg/kg body weight.

Antibodies were produced as ascites in pristane-primed BALB/C mice.[195] Ascites fluid was collected and clarified by extraction with 1,1,2-trichlorotrifluroethane[a] (0.7 ml per ml ascites). The lipid-free layer was recovered after centrifugation (1500 X g, 4 C, 10 min) and stored at -80 C. Extracted ascites fluid was diluted 1:8 in binding buffer (0.01 M sodium phosphate/0.15 M NaCl, pH 7.0), filtered (0.45 μm), and the antibody removed by repeated passage over a 25-ml protein G affinity column[b], then eluted in 0.5 M ammonium acetate, pH 3.0. Fractions with peak absorbance at 280 nm were collected into tubes containing 1 M Tris HCl, pH 9.0 (final concentration 0.07 M). These fractions were pooled and dialyzed extensively against phosphate-buffered saline solution (PBSS). All steps were performed at 4 C.

Antibody purity was evaluated by SDS-PAGE and Coomassie blue staining[c] which revealed 2 bands running identical to

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[a]Sigma Chemical Co, St, Louis, MO.

[b]Gamma-bind Plus Sepharose, Pharmacia LKB biotech, Upsalla, Sweden

[c]PhastSystem, Pharmacia LKB biotech, Upsalla, Sweden.

the heavy and light chains of a concurrently electrophoresed Mu IgG standard. No other band was seen. Protein concentration of the purified mAb solution was determined by bicinchoninic acid method.[d] Endotoxin content was determined by *Limulus amebocyte* lysate gel clot assay[e] according to guidelines prepared by the Food and Drug Administration.[204,205] Neutralizing activity of the mAb for Eq TNF was determined by adding 0.05 ml of a 1/32 dilution of TNF-rich horse serum (previously shown to contain 7500 units (U) of TNF activity per ml serum--see chapter 3) to equal volume of serial 2-fold antibody dilutions, then measuring residual cytolytic activity by standard bioassay. Neutralizing activity was calculated as follows:

$$\frac{A \times B(U/ml)}{C(mg/ml)} = D\ (U/mg)$$

where "A" is the mAb dilution factor that reduced the TNF titer to 1 RU/ml, "B" is the actual amount of TNF neutralized at that antibody dilution (control TNF titer - 1), "C" is the total protein concentration of the mAb solution, and "D" is the neutralizing activity of the mAb.

Endotoxin challenge. Endotoxin stock, as bacterial lipopolysaccharide (LPS; *E. coli* O55:B5[a]), 1 mg/ml in pyrogen-free water, was aliquoted and stored at -20 C. The morning of each experiment, an aliquot was thawed and

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[d]Pierce BCA Protein Reagent Assay, Pierce, Rockford, IL.

[e]Associated of Cape Cod, Woods Hole, MA.

diluted into 10 ml sterile 0.09% saline solution to provide a dose of 0.25 μg LPS/kg body weight. The calculated dose was administered IV over 5 min, beginning 5 min after the end of antibody treatment.

Experimental design. Male and female horses were sorted randomly into 2 groups (each with 4 males and 1 female) to receive either anti-TNF mAb or control mAb. Experiments were performed daily for 5 consecutive days on pairs of horses, one from each group. After overnight acclimation of horses in stalls, 14-gauge, 2-inch teflon catheters[f] were secured in both jugular veins. One was used for infusions and the other for blood sampling. Catheters were kept heparinized between uses and 1.5 ml air was injected to clear catheters prior to each sample collection. Antibody treatments were begun 1 h after catheterization, then LPS was given 5 min after completion of antibody infusion. During infusions, horses were kept tied. For the remainder of the experiment horses were free in the stalls with ad libitum access to water and costal Bermuda hay, and were restrained only during sample and clinical data collection. Immediately after the 24-h time-point, horses were given flunixin meglumine[g], 1 mg/kg, to mitigate any remaining effects of LPS, and returned to pasture under

[f]Abbocath, Abbott Hospitals Inc, Chicago IL.

[g]Banamine, Schering Co, Kenilworth, NJ.

observation. Follow-up blood sampling and examination was conducted approximately 1 and 2 weeks later.

Data and sample collection. Horses were monitored continuously for 12 h, and clinical signs were recorded at -0.33, 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, and 24 h after LPS. Observers, blinded as to treatment, monitored heart and respiratory rates and rectal temperatures. A single blinded observer graded attitude, gastrointestinal borborygmi, mucous membrane character, and capillary refill time (CRT) according to a semi-objective scale. These parameters were each graded as either 1, 2, 3, or 4, according to the scoring system shown in table 5-1, page 75. These scores were then combined to generate a clinical abnormality score (CAS). Time spent recumbent was recorded for each horse for 8 h after LPS was given.

At -0.33, 0, 0.5, 1, 1.5, 2, 3, 4, 6, 12, and 24 h after LPS was given, blood was collected into evacuated tubes[h] containing either no anticoagulant (Mu IgG assay), potassium EDTA (TP, WBC, and PCV determinations), or lithium heparin (TNF assay). Blood was collected by jugular venipuncture into evacuated tubes without anticoagulant at 1- and 2-week follow-up visits. Anticoagulated tubes were kept on ice less then 2 h until WBC count, PCV, and plasma (TP) concentration were determined (potassium EDTA-containing tubes), or plasma was harvested by centrifugation

[h]Vacutainer, Becton Dickinson, Rutherford NJ.

(200 X g, 4 C, 10 min, lithium heparin-containing tubes). Plain tubes were allowed to clot at room temperature, then kept at 4 C for 12 h until serum was harvested by centrifugation (200 X g, 4 C, 10 min). Serum and plasma samples were divided into 1-ml aliquots, kept on ice for less then 8 h, then stored at -80 C until assay.

Hematology. Microhematcrit centrifugation and plasma refractometry were used to determine PCV and TP concentration. Commercially available dilution/lysing chambers[i] were used to prepare blood for WBC determinations, which were made using a hemocytometer. These measurements were made within 2 h after sample collection.

TNF assay. TNF was measured in thawed plasma samples as cytolytic activity for actinomycin-D primed L929 cells (as described in chapter 3), within 2 weeks after sample collection.

Murine IgG determinations: Murine IgG concentrations were measured in thawed serum samples by ELISA. Except where noted, all steps were performed at 37 C, and volumes added were 0.1 ml/well. For washes, wells were filled with buffer (0.45 ml tris-buffered saline/surfactant/0.01% thimersol[j]), flicked, and tapped on a paper towel. ELISA plate[k] wells were coated at 4 C, overnight with Eq anti-Mu

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[i]Unopet, Becton-Dickinson, Rutherford, NJ.

[j]Perseptive Diagnostics, Cambridge, MA.

[k]Nunc Maxisorp, Marsh Biomedicals, Rochester, NY.

IgG[l] (2 μg/ml in coating buffer--0.05 M carbonate/0.15 M NaCl/0.2% sodium azide, pH 9.0), washed, blocked (0.45 ml 0.25% bovine serum albumin/0.05% Tween 20 in coating buffer), then samples or standards (HL262, 0 - 1000 ng/ml blocking buffer) were added in duplicate. Plates were washed 4X, and alkaline-phosphatase-conjugated secondary antibody[l] (Eq anti-Mu IgG-alk phos, 1/2000 in blocking buffer) was added to all wells except substrate blanks. Plates were incubated, then washed 6 times. Substrate solution (pNPP[n], 1 mg/ml in 10% diethanolamine/0.5mM $MgCl_2$/0.02% azide, pH 9.8) was added to all wells. After 30 min, 0.05 ml 0.2 N NaOH was added to stop color development. Absorbance was measured at 405 nm using an automated microtitration plate reader and software.[m] On each assay plate, a standard curve and a substrate blank were included. Duplicate measurements were averaged and blank absorbances were subtracted from all values. Sample Mu IgG concentrations were interpolated from the linear portion of the Mu IgG standard curve by regression. Sample concentrations fell within the linear range of the standard curve (25 - 250 ng/ml), with the exception of those obtained at -0.33 h (both groups) and at 2 weeks after LPS was given (anti-TNF-treated group). These were below the limit of

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[l]H+L polyclonal, Vector Laboratories, Burlingame, CA.

[m]Titertek Multiskan MC, Titersoft II, Flow Laboratories LTD, Irvine, Scotland.

detection. The ELISA sensitivity was calculated as that Mu IgG concentration with an absorbance equal to the mean background absorbance + 2 standard deviations (SD), (n=8). Intra-assay variance was calculated from a single sample assayed 8 times as the (SD/mean) * 100.

Statistical analysis. All continuously variable data were analyzed by 2-factor ANOVA (with treatment a fixed effect and time a repeated measure). Significant effect of treatment was revealed by time X treatment interaction P-values of < 0.05. If treatment effects were demonstrable, pairwise comparisons at each time-point were explored by Tukey's Honest Significant Difference test. Prior to analysis, TNF data were logrithmically transformed to minimize heterogeneity of variance. Unless otherise stated, data are reported as mean ± SEM. Ranked ordinal data (clinical abnormality scores) were compared between treatment groups at each time-point by the non-parametric Mann-Whitney U test. All manipulations were made using a commercially available statistics software program.[n]

Results

Antibody preparations. The anti-TNF antibody, HL801, had a neutralizing titer for Eq TNF of 1.5×10^5 U/mg, while the control antibody had no detectable activity against Eq TNF. A trace level of endotoxin was detected in both

[n]Statistica, StatSoft Inc, Tulsa, OK.

control (0.56 - 1.1 ng endotoxin/mg antibody) and anti-TNF (0.86 - 1.7 ng endotoxin/mg antibody) antibody solutions. The total dose of LPS given from endotoxin contamination of antibody treatments was between 1.0 to 2.0 ng/kg in control horses, and between 1.6 to 3.1 ng/kg in anti-TNF-treated horses. This can be compared to the 250 ng/kg dose of LPS given after antibody infusion.

Clinical signs. After IV administration of endotoxin, all horses in the control group exhibited transient depression, colic, anorexia, tachycardia, tachypnea, injected mucous membranes, and pyrexia. In contrast to control horses, anti-TNF-treated horses had significantly ($P < 0.010$) lower clinical abnormality scores, lower heart rates, and spent less time in recumbency.

Clinical abnormality score data (combined scores of attitude, borborygmi, mucous membrane character, and CRT) are depicted in figure 5-1. In the control group, highest scores (median 12, range 9 - 14) were seen at 3 h after LPS administration, and scores remained elevated for 12 h after LPS was given. In the anti-TNF-treated group, CAS reached a maximal median value of 7 (range 4 - 13) 1.5 h after LPS was given, and quickly returned to baseline. Scores from the anti-TNF-treated group were significantly lower then control group CAS at 2, 3, 4, 6, 8, and 12 h after LPS was given.

The effect of anti-TNF treatment on heart rate was highly significant ($P < 0.001$). In control horses, heart

rates from rose from 48 ± 3.6 beats/min to 73.6 ± 3.2 beats/min 2 h after LPS was given (figure 5-2). After a trend towards baseline after 2 h, horses in this group experienced another rise in heart rate, which was sustained for the duration of the experiment (24 h). In contrast, heart rates in anti-TNF-treated horses rose only slightly at 2 h then declined to baseline values for the remainder of the observation period. Significant differences between groups were detected at 1.5, 2, 6, 8, 12, and 24 h post-LPS administration.

A significant effect of anti-TNF treatment on respiratory rate was not detected ($P = 0.250$) (fig 5-3). Horses in both groups exhibited biphasic tachypneic response with peaks at 0.5 h and 4 to 6 h after LPS was given. During the late-phase tachypnea, there was a trend towards lower respiratory rates in anti-TNF treated horses.

There was also no significant ($P = 0.350$) effect of treatment on the febrile response to LPS (fig 5-4). Temperatures climbed steadily from baseline to maximal values 4 h after LPS was given, then gradually returned to baseline by the end of the experiment.

In the control group, 4 of 5 horses became recumbent after LPS administration, with a total recumbency time for this group of 294 min. Two of five anti-TNF treated horses became recumbent, with total recumbency time of 33 min. Interestingly, horses given anti-TNF were recumbent only

during the first hour after LPS was given, while 286 of the 294 minutes of recumbency in the control group occurred between 1 and 4 hours after LPS.

Hematology. There was a highly significant ($P < 0.001$) effect of treatment on WBC count. In both groups, WBC count dropped quickly after LPS administration (fig 5-5). However, minimal values in the anti-TNF treated group were higher then those in the control group, and quickly rebounded to baseline values by 3 h after LPS was given. Leukopenia in the control group was sustained for at least 6 h. Differences were significant at 3, 4, and 6 h after LPS was given. There was no significant effect of treatment on PCV ($P = 0.073$, figure 5-6) or plasma TP concentration ($P = 0.440$, figure 5-7).

Tumor necrosis factor. Anti-TNF treatment almost completely neutralized LPS-induced circulating TNF activity (fig 5-8). This effect of anti-TNF was highly significant ($P < 0.001$): mean TNF activity was significantly lower in anti-TNF-treated horses at 1, 1.5, 2, 3, 4, and 6 h after LPS was given. Horses in the control group experienced the predictable monophasic rise of circulating TNF activity, obtaining maximal mean value (4,179 $\pm$ 2,712 U/ml) at 1.5 h after LPS was given.

Serum murine IgG concentration. The ELISA had sensitivity of 28 ng/ml and intra-assay variance of 12.2%.

The standard curve was linear (R^2 = 0.99) for a range of Mu IgG concentrations between 25 and 200 ng/ml.

Measured serum Mu IgG was maximal 35 min after the end of antibody infusion (fig 5-9, 67.1 ± 4.8 μg/ml and 66.7 ± 4.3 μg/ml for anti-TNF- treated and control-treated groups respectively). Murine antibody remained at high concentration in the serum of both groups for the duration of the experiment. Low concentration of antibody was still detected in serum from horses in both groups at 7 days, and from horses in control group at 14 days.

Discussion

Treatment with anti-TNF mAb at a dose of 1.86 mg/kg almost completely neutralized circulating TNF activity in this model. This mAb dose (or possibly lower doses) should therefore be more then sufficient to block TNF during clinical endotoxemia because the circulating activities of this cytokine are much lower in horses with naturally occurring diseases.[21,23,24]. Reported circulating TNF activities in horses with enteric diseases and foals with presumed sepsis were less then 200 U/ml.[21,23,24]

The amount of LPS (1 - 4 ng/kg) given as contaminant with mAb may, by itself cause a slight febrile response in horses.[106] We therefore chose to administer the mAb treatments immediately before giving the major LPS challenge in order to prevent a possible tolerizing effect. It should

be noted that the level of contamination was slightly higher in the anti-TNF mAb solution then it was in the control mAb solution. Thus, if the LPS in mAb treatments had incremental effect, such effect should bave been more pronounced in horses given the anti-TNF mAb.

Fever is one of the most consistent changes induced by LPS administration at all dosages. Endotoxin and/or mediators produced during endotoxemia appear to influence the hypothalamic thermoregulatory center, causing fever.[206-208] The role of TNF in LPS-induced pyrexia is not clear. Infusion of TNF has been shown to cause fever, however TNF blockade did not affect the pyrexic response in mice, rats, or rabbits given LPS.[131,132-140,146,206] Rats given anti-TNF antiserum 2 h before LPS was given experienced higher fevers then rats pretreated with normal serum.[208] Conversely, 2 studies reported significantly lower temperatures in anti-TNF treated rabbits given LPS.[207,209] In our study, treatment with mAb to TNF did not reduce fever in horses given LPS, indicating that endotoxin, or other endogenous factors mediate this response in the equine species.

In other species, TNF blockade protected against mortality and morphologic organ damage, attenuated metabolic, hypotensive, and pro-coagulatory responses, but did not block production of thromboxane and associated elevations in pulmonary arterial hypertension seen after LPS administration.[149-161]. Thromboxane production occurs within

1 h after LPS and induces pulmonary arterial hypertension.[54] The early-phase tachypnea seen in both anti-TNF-treated and control-treated horses could be due to thromboxane, while the late-phase tachypnea may be due, in part, to pyrexia. These explain why TNF blockade did not have a significant effect on LPS-induced tachypnea.

Endotoxin-induced tachycardia, depression, colic, reduction in ascultable intestinal sounds, prolonged capillary refill time, and mucous membrane injection, all were significantly improved in anti-TNF-treated horses as compared to control-treated horses from 2 to 12 h after LPS was given. Circulating TNF activity is maximal 1.5 h after LPS is given, so it is not surprising that improvements seen with TNF blockade occur after this time. These results confirm that TNF is a central mediator of the adverse clinical signs of equine endotoxemia.

Endotoxin, along with cytokines and inflammatory mediators released during endotoxemia, induces expression of neutrophil surface glycoproteins, and promotes adherence of neutrophils to the vascular endothelium.[49,50,51] Vascular endothelial adhesion molecules for granulocytes are also up-regulated during endotoxemia.[50,52,53] A profound leukopenia and neutropenia results within 1 h after LPS is given.[85,89,90,91] In previous studies, anti-TNF mAb given in models of endotoxemia or sepsis did not completely prevent, but did lessen the severity of LPS-induced leukopenia.[152,156]

This is in accordance to the results of this study, where WBC count in horses treated with anti-TNF were significantly higher then those of control-treated horses at 3, 4, and 6 h after LPS was given. On the basis of these results, the initial leukopenic response of horses to LPS does not appear to be entirely TNF-mediated, but TNF does appear to be an important factor in a sustained leukopenic response.

Increases in PCV after LPS administration have been dose-dependent in models of equine endotoxemia previously reported: administration of 100 μg LPS/kg produced marked elevation in hematocrit; 10 μg LPS/kg produced minor elevations in hematocrit; and 30 ng LPS/kg had no effect on hematocrit.[84,85,90,108] Splenic contraction has been implicated as a likely cause of LPS-induced elevations in hematocrit.[90] In our study, there was a small elevation in PCV 2 h after LPS was given in control-treated horses; however, significant effect of mAb treatment was not seen. Significant effect of anti-TNF on PCV may only be apparent when higher doses of LPS are given. Changes in plasma protein concentrations have not been associated with LPS administration.[85,91,103] As expected, there was also no significant effect of mAb treatment on plasma TP concentration in our study.

Murine monoclonal antibodies against recombinant-derived human TNF have been produced (CB0006 and Bay x 1351) and are currently being tested in prospective, double-blind,

placebo-controlled studies of humans patients with sepsis syndrome.[159,160,210] Safety and pharmacokinetic studies of CB0006 and Bay x1351 have shown that adverse reaction to these products is rare, despite development of anti-murine IgG antibody in the majority of mAb-treated patients.[160,210] Murine anti-TNF mAb was shown to have a long half-life (50 h).[160,210] This is agreement with the current study, in which serum murine mAb concentration was high (approximately 40 μg/ml) in both groups of horses 24 h after LPS was given, and was detected (approximately 7 μg/ml) one week later. Because of this extended half-life, a single dose of anti-TNF mAb potentially could neutralize circulating TNF for several days in endotoxemic patients. The clinical efficacy of anti-TNF mAb therapy in endotoxemic human patients has not yet been evaluated satisfactorily. Administration of CB0006 to human patients with unresponsive hypotensive shock and presumed septicemia caused immediate and sustained rise in mean arterial pressures.[160] In a multi-center prospective trial, 80 patients with severe sepsis or septic shock were given differing doses of CB0006 in addition to standard care.[160] Only 35 of 80 patients in this study had elevated TNF activity prior to mAb therapy, and this smaller group of patients appeared to benefit from anti-TNF treatment. However, there was no benefit of anti-TNF treatment in the overall study population.[160]

It is apparent that TNF plays a major role in responses of horses to endotoxin. In the model of endotoxemia used in our study, horses given anti-TNF mAb had significantly improved clinical abnormality scores, lower heart rates, and higher WBC counts then control-treated horses. Factors other then TNF are responsible for LPS-induced pyrexia and tachypnea. Treatment with anti-Eq TNF mAb may be useful in endotoxemic horses, particularly in those patients where circulating TNF activity is present.

Table 5-1. Scoring of abnormal clinical signs in horses given endotoxin.

Score	Clinical Appearance	Mucous Membranes	Capillary Refill Time	Gut Sounds
1	bright and alert, normal appetite	pink, moist	< 2.0 sec	> 5/3 min
2	mild depression and anorexia, +/- mild colic	slightly dry, pale or dark pink	2 - 3 sec	3-5/3 min
3	moderate depression and anorexia, +/- bouts of sternal recumbency, +/- mild colic	dry or slightly tacky, dark pink to red	3 sec	1-2/3 min
4	marked depression, frequent sternal recumbency and occasional lateral recumbency	dry and tacky, brick red with toxic rim	> 3 sec	0/3 min

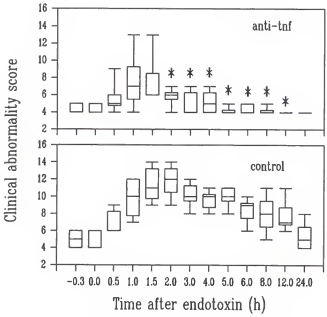

Figure 5-1. Effect of anti-TNF on clinical abnormality score (aggregate score for attitude, GI borborygmi, mucous membrane character, and CRT) in horses given LPS. Horizontal bars within boxes are medians, box margins are 25th and 75th percentiles, and error bars are 10th and 90th percentiles. Asterisks mark significant differences between groups at indicated time-points ($P < 0.010$, n = 5)

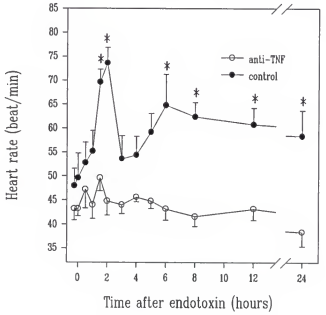

Figure 5-2. Effect of anti-TNF on heart rate of horses given LPS. Data are shown as mean ± SEM. Asterisks mark significant differences between groups at the indicated time-points ($P < 0.010$, n = 5).

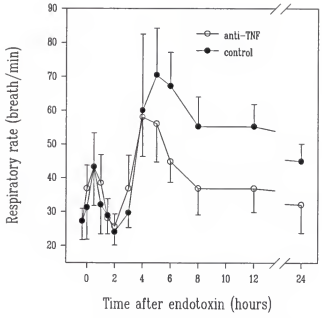

Figure 5-3. Effect of anti-TNF on respiratory rates of horses given LPS. Data are shown as mean ± SEM. A significant effect of treatment was not detected (P = 0.250, n = 5).

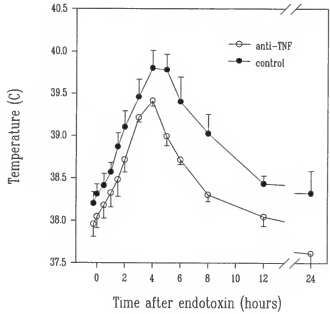

Figure 5-4. Effect of anti-TNF on rectal temperature of horses given LPS. Data are shown as mean ± SEM. A significant effect of treatment was not detected (P = 0.350, n = 5).

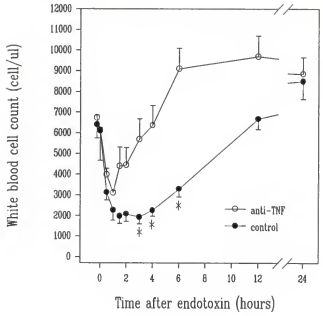

Figure 5-5. Effect of anti-TNF on white blood cell count of horses given LPS. Data are shown as mean ± SEM. Asterisks mark significant differences between groups at indicated time-points ($P < 0.010$, n = 5).

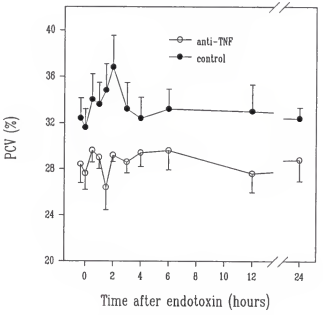

Figure 5-6. Effect of anti-TNF on packed cell volume of horses given LPS. Data are shown as mean ± SEM. A significant effect of treatment was not detected ($P = 0.073$, n = 5).

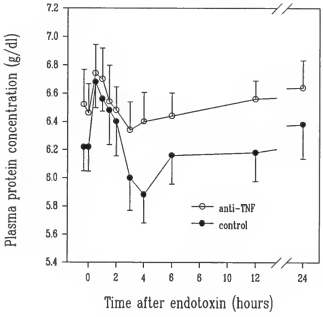

Figure 5-7. Effect of anti-TNF on plasma total protein concentration of horses given LPS. Data are shown as mean ± SEM. A significant effect of treatment was not detected (P = 0.440, n = 5)

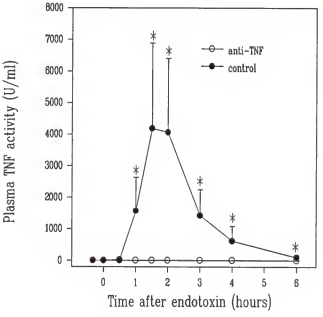

Figure 5-8. Effect of anti-TNF on circulating TNF activity of horses given LPS. Data are shown as mean ± SEM. Asterisks mark significant differences between groups at indicated time-points (P < 0.005, n = 5).

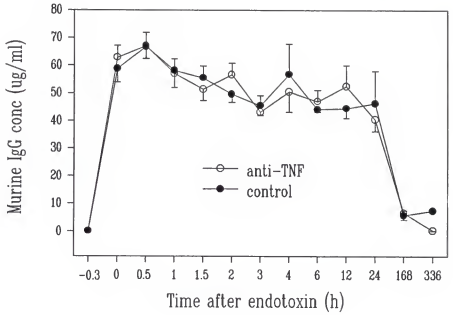

Figure 5-9. Effect of anti-TNF on serum murine immunoglobulin concentration in horses given LPS. Data are shown as mean ± SEM. A significant effect of treatment was not detected. ($P > 0.050$, n = 5)

CHAPTER 6
EFFECTS OF NEUTRALIZING TUMOR NECROSIS FACTOR ON INTERLEUKIN 6, LACTATE, THROMBOXANE, AND PROSTACYCLIN RESPONSES IN HORSES GIVEN ENDOTOXIN

Summary

A monoclonal antibody (mAb) against equine (Eq) tumor necrosis factor alpha (TNF) was used to investigate the role of TNF in cytokine, eicosanoid, and metabolic responses of horses given endotoxin. Plasma concentrations of interleukin 6 (IL-6), lactate, thromboxane A_2 metabolite (TXB_2) and prostacyclin metabolite (6-keto-$PGF_{1\alpha}$) were measured in 10 adult miniature horses given 0.25 μg lipopolysaccharide (LPS; Escherichia coli O55:B5). Five horses were given anti-Eq TNF mAb and five horses were given isotype-matched mAb as control. All horses were given 1.86 mg antibody/kg bodyweight by intravenous (IV) infusion, 5 min before LPS was given IV. Blood samples were taken 20 min prior to, and at multiple intervals for 24 hours (h) after LPS was given. Interleukin 6 bioactivity in plasma was measured using IL-6-dependent cell line (B9). Eicosanoid activities were assessed by enzyme immunoassay, and plasma lactate concentration was determined enzymatically. Data were analyzed by ANOVA and Tukey's Honest Significant Difference test for significant ($P < 0.05$) effect of

treatment. Horses given anti-Eq TNF mAb had significantly ($P < 0.050$) lower maximal mean ± SEM IL-6 (59 ± 29 U/ml), lactate (16 ± 1.80 mg/dl), and 6-keto-$PGF_{1\alpha}$ (254 ± 79 pg/ml) values then did horses given control antibody (880 ± 375 U/ml for IL-6; 26 ± 0.03 mg/dl for lactate; 985 ± 290 pg/ml for 6-keto-$PGF_{1\alpha}$). There was no effect of anti-TNF treatment on LPS-induced TXB_2 production. Tumor necrosis factor mediates IL-6, lactate, and prostacyclin responses, without affecting thromboxane production in horses given LPS.

Introduction

Many adverse effects seen during endotoxemia are caused by interaction of host-produced mediators.[4,12-15,26,33,44] Monoclonal antibodies are effective tools for defining the roles of specific mediators in the pathogenesis of LPS-induced disease.[210-212] Previously, use of anti-TNF mAb in animals models of endotoxemia significantly increased survival and supported the pivotal role of TNF in the pathogenesis of endotoxemia.[149-161] Circulating TNF activity is detected early during experimentally produced and naturally acquired endotoxemia, and correlates closely with severity of disease and rate of mortality.[132-149] Monoclonal anti-TNF antibodies are currently being evaluated clinically in human patients with sepsis syndrome.[159,160,210]

Circulating TNF activity has been detected in horses with enteric disorders and foals with presumed seticemia. In these settings, TNF activity correlated with disease severity and mortality.[21,23,25] As is the case in other species, horses produce circulating TNF activity with maximal values seen approximately 90 min after IV LPS administration.[20,101]

Other inflammatory mediators have been identified in circulation of horses given endotoxin. Interleukin 6 activity was induced, and reached maximal activity 180 min after LPS was given.[106] Plasma thromboxane concentration was highest 30 min after LPS was given, and was associated with development of tachypnea, tachycardia, elevated pulmonary arterial pressure (PAP), hypoxemia, hypocapnia, and respiratory alkalosis.[83-94] Prostacyclin metabolite reached maximal circulating concentration 120 min after LPS was given, and correlated with systemic hypotension in horses given lethal endotoxin challenge.[87,88,90,92] Treatment with flunixin meglumine reduced rises in circulating eicosanoid metabolite concentrations and attenuated associated hemodynamic alterations in endotoxemic horses, but did not have an effect on mortality or TNF responses.[87,90,98]

Documented metabolic alterations following endotoxin administration to horses include hyperglycemia followed by hypoglycemia, and elevated blood lactate concentrations.[85,86,89,92,94] Increased procoagulant activity,

disturbances in splanchnic blood flow, reduced gastrointestinal motility, and increased serum enzymes reflective of cellular injury have been reported in horses given LPS.[85-91,95,105,109] Horses given LPS exhibit leukopenia, fever, colic, and depression.[85,89-93,96-100]

Treatment with anti-TNF mAb, as compared to control mAb, significantly reduced circulating TNF activity and attenuated clinical and leukopenic responses of horses to LPS (described in chapter 5). The purpose of this investigation was to determine effects of TNF neutralization on LPS-induced mediator (IL-6, TXB_2, 6-keto-$PGF_{1\alpha}$) and metabolic (lactate) responses.

Materials and Methods

Horses and in vivo experiments. The experimental design, animals, and antibody treatments used in this investigation were described in detail in chapter 5. Briefly, 10 adult miniature horses were used. The treatment group was 5 horses given murine monoclonal antibody (mAb) against Eq TNF. This mAb neutralized 150,000 U of Eq TNF per mg antibody. The control group was five horses given an isotype-matched mAb that had no activity for Eq TNF. Both antibodies were produced as ascites in pristane-primed BALB/c mice then protein G-purified.

Experiments were performed daily for 5 consecutive days on pairs of horses, one from each group. Antibodies were

diluted into 100 ml sterile non-pyrogenic 0.9% saline solution and given via jugular catheter at total dosage of 1.86 mg/kg bodyweight over 15 minutes (min). Five min after the end of antibody infusion (experimental time = 0), horses were given 0.25 μg endotoxin (LPS; E. coli 055:B5[a])/kg, IV in 10 ml sterile saline solution, over 5 min.

Sample collection. Horses were observed continuously for 12 hours (h), and venous blood was collected -0.33, 0, 0.5, 1, 1.5, 2, 3, 4, 6, 12, and 24 h after LPS was given into evacuated tubes[b] containing lithium heparin (IL-6 assay) or Na fluoride (lactate assay). Plasma was harvested by centrifugation (200 X g, 4 C, 10 min) less then 2 h after blood collection, aliquoted into sterile polyproplyene tubes, and stored at -80 C until assay. All samples were kept on ice during processing.

For eicosanoid determinations, venous blood was collected -0.33, 0, 0.5, 1, 2, 4, and 6 h after LPS was given into chilled syringes containing meclofenamic acid[c] (MFA) and EDTA (final concentration 0.5 μM MFA/5 μM EDTA). After gentle mixing, the anticoagulated blood was immediately transferred to chilled polypropylene centrifuge tubes and kept on ice for less then 30 min until plasma was harvested by centrifugation (1800 X g, 4 C, 20 min).

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[a]Sigma Chemical Co, St Louis, MO.

[b]Vacutainer, Becton Dickinson, Rutherford, NJ.

[c]Warner Lambert Co, Ann Arbor, MI.

Aliquots of plasma were stored at -80 C for 12 - 15 weeks until assay.

Assay for Interleukin 6. Plasma IL-6 activity of all samples were quantified in a single assay 3 weeks after collection as the ability to promote growth of the IL-6-dependent B-cell hybridoma B13.29, clone B9 (B9).[107] Cell lines were maintained as previously described.[107] Assay medium was RPMI 1640[a] supplemented with 2 mg/ml $NaHCO_3$, 2 mM glutamine[a], 100 U/ml potassium penicillin G[a], 100 μg/ml streptomycin[a], 15 mM HEPES buffer[a], 0.1 mM non-essential amino acids[d], 20% (v/v) fetal bovine serum[a], and 50 μM 2-mercaptoethanol.[a] Fifty microliter amounts of sample diluted 1/10 or 1/20 were pipetted in triplicate into microtitration plates containing 50 μl assay medium/well. Assay medium was added to negative control wells and recombinant-derived human IL-6[e] sufficient to cause maximal B9 proliferation was added to positive control wells. Serial 2-fold dilutions of samples were made and a suspension of B9 cells (2×10^5 cells/ml) was added to all wells (50 μl/well) and plates were kept for 44 h at 37 C in 5% CO_2 in air. After incubation, 25 μl of 3-(4,5-dimethylthiazole-2-yl)-2,5-triphenyl-tetrazolium bromide,[a] 4 mg/ml in phosphate-buffered saline solution (PBSS), was added to all wells, and the plates were incubated for an additional 4 h. Finally,

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[d]GIBCO Laboratories, Grand Island, NY.

[e]Boehringer Mannhiem Co, Indianopolis, IN.

100 μl of 2-propanol/0.4 N HCl was mixed into each well and the optical density at 540 nm was determined using an automated microtiter plate reader and software.[f] By convention, the sample dilution that caused exactly 50% maximal cellular proliferation as compared to positive control wells contained 1 U IL-6 activity/ml.

Assay for lactate. Plasma lactate concentrations were quantified in a single assay by an enzymetric method using commercially available kits[g]. In this assay, oxidation of sample lactate to pyruvate and reduced nicotinamide adenine dinucleotide (NADH) is catalyzed by addition of excess lactate dehydrogenase (LDH) and NAD^+. The increased absorbance of samples at 340 nm due to NADH formation is measured as a function of lactate originally present in the sample. After 6 weeks storage, plasma samples were thawed and deproteinated by addition of 0.4 ml cold 10% trichloroacetic acid[a] to 0.2 ml plasma, followed by centrifugation (1500 X gravity, 4 C, 10 min). After centrifugation, 0.1 ml of supernate were assayed in duplicate according to kit manufacturer's instructions. Duplicate measurements were averaged, blank absorbances were subtracted from all values, and sample lactate

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[f] Titertek Multiskan MC, Titersoft II, Flow Laboratories LTD, Irvine Scotland.

[g] Procedure #826-UV, Sigma Diagnostics, St Louis, MO.

concentrations were interpolated from the linear portion of the lactate standard curve by regression analysis.

Assay for thromboxane metabolite (TXB_2). Two-milliliter plasma samples were acidified to pH 3 - 4 in 10 ml borosilicate centrifuge tubes by addition of 0.2 ml 1 N HCl. The eicosanoid fraction was extracted from acidified plasma by addition of 6 ml ethyl acetate. Tubes were capped, inverted 15 times, allowed to sit undisturbed for 30 - 60 min, then supernate was decanted into 13 X 100 mm disposable borosilicate culture tubes and dried under a stream of nitrogen gas. Aliquots of normal horse plasma (NHP) were spiked with TXB_2 standard solutions[h], (0 pg/ml and 385 pg/ml final concentration) then extracted and assayed with samples to determine extraction efficiency.

Competitive enzyme immunoassay kits[h] were used to measure the stable metabolite of thromboxane A_2 (TXB_2) in extracted samples according to manufacturer's instructions. Except where noted, incubation temperature was 4 C and volumes added were 0.1 ml/well. Extracted, dried samples were reconstituted into 0.25 ml PBSS and added in duplicate to wells of polystyrene ELISA strips coated with goat anti-rabbit capture antibody. Thromboxane B_2 standards (0, 50, 250, 1000, and 5000 pg/ml) also were added in duplicate. Rabbit anti-TXB_2 antibody was added to all wells except substrate blanks, and strips were incubated overnight.

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[h]Titerzyme, Perseptive Diagnostics, Cambridge MA.

Alkaline phosphatase-conjugated TXB_2 was added (all wells except substrate blanks) and incubated for 3 h. Strips were washed 3 times by sequential addition and removal of 0.4 ml wash solution, then 0.05 ml substrate solution was added to all wells and the strips were incubated for 1 h at 37 C. Color development was stopped by addition of 0.05 ml of 0.2 N NaOH, and absorbance was measured at 405 nm using an automated microtitration plate reader and software.[f] On each assay plate, a standard curve, a substrate blank, and high and low-spiked NHP samples (NHP-0, NHP-385) were included with research samples. Blank absorbances were subtracted from all values and sample concentrations were calculated from the linear portion of the standard curve by regression analysis. Extraction efficiency was 70% as determined from NHP-0 and NHP-385 samples.

Assay for prostacyclin metabolite (6-keto-$PGF_{1\alpha}$). For measurement of 6-keto-$PGF_{1\alpha}$, samples were handled exactly as for TXB_2 except that the concentrations of metabolite for the standard curve were 0, 10, 50, 250, 1000, and 5000 pg/ml. Extraction efficiency for this metabolite was 84%.

Statistical Analysis. All continuous data were analyzed by 2-factor ANOVA (with treatment a fixed effect and time a repeated measure). Significant effect of treatment was revealed by time X treatment *P*-values of < 0.050. If treatment effect was demonstrable, pairwise comparisons at each time-point were explored post-hoc by

Tukey's Honest Significant Difference test. Prior to analysis, IL-6 data were transformed logarithmically to minimize heterogeneity of variance. All manipulations were made using a commercially available statistics software program.[i] All data are reported as mean ± SEM unless stated otherwise.

Results

Interleukin 6. Horses given control antibody had maximal plasma IL-6 activity 4 h after LPS administration of 880 ± 375 U/ml (figure 6-1). Horses given anti-TNF had much smaller IL-6 response with maximal IL-6 activity of 59.2 ± 29 U/ml. This effect was highly significant ($P < 0.001$), and significant effect of treatment was present at 2, 3, 4, and 6 hours after LPS was given.

Lactate. There was a small but significant ($P < 0.005$) effect of treatment on plasma lactate concentration. At 3 h after LPS was given, lactate values were 26 ± 0.04 mg/dl vs 16 ± 2.00 mg/dl for control and anti-TNF-treated groups, respectively (fig 6-2). Pairwise comparison revealed significant difference between groups at 2, 3, and 4 h after LPS was given.

Thromboxane metabolite (TXB_2). As shown in figure 6-3, both groups of horses exhibited similar monophasic peaks of plasma TXB_2 activity after LPS was given. Maximal mean

[i]Statistica, StatSoft Inc, Tulsa, OK.

values of 2095 ± 299 pg/ml and 1954 ± 224 pg/ml were recorded at 0.5 h after LPS administration for control and anti-TNF-treated horses, respectively. This effect was not significant ($P = 0.659$).

Prostacyclin metabolite (6-keto-$PGF_{1\alpha}$). Control horses responded to LPS with a single peak of plasma 6-keto-$PGF_{1\alpha}$ activity 2 h after LPS was given (figure 6-4). By comparison, this response was significantly ($P < 0.001$) abrogated in anti-TNF-treated horses. Maximal plasma concentration (985 ± 290 pg/ml) was detected in control horses 2 h after LPS was given. This is compared to maximum plasma concentration of 254 ± 79 pg/ml detected in anti-TNF-treated horses 0.5 h after LPS administration. Significant difference between groups was found at the 2-h time-point.

Discussion

Treatment with anti-TNF mAb almost completely neutralized LPS-induced circulating TNF activity in these horses (chapter 5). Because control horses were given isotype-matched antibody, specific effects of TNF blockade are the only plausible explanation for the observed differences between groups. Horses treated with anti-TNF had significantly improved clinical appearance compared to control-treated horses (chapter 5). This indicated that TNF, or mediators induced by TNF, have significant clinical effect in horses.

Interleukin 6 is a potent stimulator of the acute-phase response during endotoxemia. Monoclonal antibodies against IL-6 did not protect mice from LPS-induced mortality, and IL-6 infusion had an additive protective effect when combined with TNF mAb therapy during murine endotoxemia.[158] These observations imply that IL-6 may have a protective, rather then causative role in the pathophysiology of endotoxemia.

In the present study, IL-6 activities were reduced significantly by neutralization of TNF. This is in general agreement with studies in rats and baboons, with some distinctions. Baboons were given live Escherichia coli which rapidly caused lethality in animals not treated with anti-TNF.[151] This design induced much greater circulating IL-6 activity then our study, and anti-TNF mAb given less then 2 h prior to sepsis only partially reduced the IL-6 response. In another study, rats were given 30 mg/kg of anti-TNF mAb 4 h before LPS was given.[149] This completely neutralized circulating TNF activity, but only partially suppressed circulating IL-6 activity.[149] In work reported here, horses given anti-TNF mAb 5 min before LPS had a highly significant reduction in IL-6 activity. The variation in results from these studies could be due to model or species differences.

Neutralization of TNF activity had variable effects on eicosanoid production in horses given LPS. Thromboxane A_2

causes vasoconstriction, platelet aggregation, and, in some studies, increased vascular permeability.[54,58-60] Treatment with anti-TNF mAb did not reduce thromboxane production in horses given LPS. A similar observation was made in a porcine model of gram-negative sepsis where pigs given anti-TNF mAb immediately before the onset of sepsis had attenuated metabolic acidosis and less extravascular lung water then untreated controls, but pulmonary arterial hypertension and thromboxane production were not affected.[153] Thromboxane activity peaks before TNF activity, so it is not surprising that TNF neutralization does not impact thromboxane production. Tachypnea seen within 1 h of LPS administration in horses was not affected by anti-TNF treatment. Elevated circulating thromboxane activity probably mediates this response by causing pulmonary arterial constriction and hypoxemia. This may explain why TNF blockade had no effect on tachypnea.

Prostacyclin is a potent vasodilator and inhibitor of platelet aggregation.[54,58-60] Elevated circulating activities of prostacyclin have been associated with systemic hypotension in horses given LPS, and flunixin administration prevented eicosanoid production and hemodynamic alterations. However, prostacyclin has also been shown to improve microcirculation, particularly in the splanchnic bed, and prevent tissue hypoxia during endotoxemia.[54,60,213,214] Evidently, prostacyclin has both protective and detrimental

effects during endotoxemia, possibly depending on the amount produced. Tumor necrosis factor is an important stimulus for prostacyclin production: neutralization of TNF significantly prevents LPS-induced increases in plasma prostacyclin concentrations, and TNF infusion causes prostacyclin production.[137] In human patients with unresponsive hypotension and presumed septicemia, administration of anti-TNF mAb caused an immediate and sustained rise in arterial pressure.[159] Reduced prostacyclin production may have been responsible for this effect.

Latacte production during endotoxemia has been attributed to tissue hypoxia with a subsequent shunt to anaerobic metabolism.[81,82] Macrophages and other inflammatory cells stimulated with LPS have also been shown to produce lactate in vitro, and may contribute to its production in vivo.[215] When horses were given much more LPS (100 μg/kg) then that given in this study (0.25 μg/kg) hypotensive shock and progressively worsening lactic acidosis resulted.[85,85,89,91,94] Hypotensive shock was obviously not induced in the current study, however hemodynamic measurements were not taken to evaluate more subtle blood flow alterations. At doses of 30 ng/kg, LPS has been shown to reduce splanchnic blood flow and inhibit gastrointestinal motility.[104,105,109] Regardless of the source of plasma lactate, TNF appears to play a significant role in its production.

In conclusion, TNF induces IL-6 and prostacyclin production in horses given LPS, as demonstrated by significantly less production of these mediators in anti-TNF-treated horses. A small increase in plasma lactate produced by sublethal LPS administration also was attenuated by prior administration of anti-TNF mAb. Thromboxane production was not affected. Early phase tachypnea seen in anti-TNF-treated and control-treated horses given LPS likely is due to thromboxane. Treatment with anti-TNF mAb appears to attenuate many responses of horses to LPS and may be beneficial in endotoxemic patients.

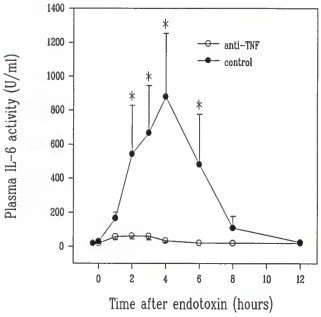

Figure 6-1. Effect of anti-TNF on plasma interleukin 6 activity of horses given LPS. Data are shown as mean ± SEM. Asterisks mark significant differences between groups at indicated time-points ($P < 0.005$, n = 5).

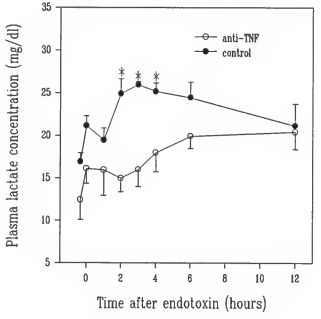

Figure 6-2. Effect of anti-TNF on plasma lactate concentration of horses given LPS. Data are shown as mean ± SEM. Asterisks mark significant differences between groups at indicated time-points ($P < 0.050$, n = 5).

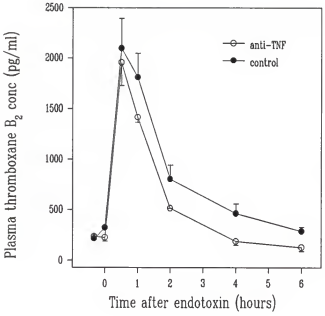

Figure 6-3. Effect of anti-TNF on plasma thromboxane metabolite (TXB_2) concentration in horses given LPS. Data are shown as mean ± SEM. A significant effect of treatment was not detected (P = 0.659, n = 5)

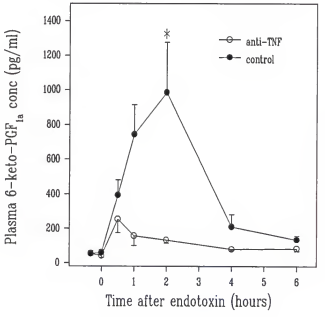

Figure 6-4. Effect of anti-TNF on plasma prostacyclin metabolite (6-keto-$PGF_{1\alpha}$) concentration in horses given LPS. Data are shown as mean ± SEM. The asterisk marks significant difference between groups at the indicated time-point (P < 0.001, n = 5).

CHAPTER 7
GENERAL DISCUSSION AND FUTURE DIRECTIONS

The goal of these investigations was to prove the hypothesis that "TNF plays a significant role in the responses of horses to endotoxin". Experiments described in chapters 3 and 4 developed necessary reagents while those described in chapters 5 and 6 tested the hypothesis. The *in vivo* experiments were designed carefully to ensure that differences seen between groups of horses given endotoxin were due entirely to a significant reduction in circulating TNF activity. Prior to LPS administration, horses received antibody treatments which differed only in activity against TNF and amount of endotoxin contamination. The slightly dissimilar amount of LPS in treatments was not a confounding factor, as it was approximately 100-fold less then the challenge dose of LPS given immediately after antibody treatment. The horses were closely-matched (same breed, same sex, similar age), and a horse from each group (anti-Eq TNF and control) was studied on each experimental day to avoid horse or environmental conditions being confounding factors.

The first observation crucial to proving the experimental hypothesis was that circulating TNF activity

was almost completely neutralized in anti-TNF-treated horses while control-treated horses responded to LPS administration with production of circulating TNF activity. This verified that differences between groups were due to significant TNF neutralization. The variables that were evaluated reflected many aspects of host responses to endotoxin--clinical, cytokine, hematologic, prostanoid, and metabolic. The observation that horses given anti-TNF antibody had significant improvement in all categories of response variables when compared to control-treated horses proves that Eq TNF production mediates many responses of horses to endotoxin.

Anti-TNF treatment neutralized circulating TNF, IL-6, and prostacyclin activities without affecting LPS-induced fever, tachypnea, and thromboxane production. This indicates that TNF, IL-6, and prostacyclin are not mediators of these responses. Thromboxane induces pulmonary arterial hypertension and arterial hypoxemia, and is a likely cause of early tachypneic responses of horses to LPS.[54] Thromboxane production may be directly induced by LPS as maximal circulating levels of thromboxane metabolite are present within 30 min after LPS administration--before TNF, IL-1, and IL-6 activities are increased.[2,34,37,54] Fever is consistently produced in response to endotoxemia, and may be a major factor in producing tachypnea from 4 - 12 h after

LPS is given.[206-208] Interleukin 1 was not measured in this experiment and may be a factor in LPS-induced fever.[33,34,41]

This model of equine endotoxemia reproduces many clinical and hematologic symptoms seen in horses suffering from endotoxin-associated conditions. Certain aspects are different, however, such as the higher circulating activities of TNF produced and the relatively short course of detectable clinical aberrations. Development of chronic models of endotoxemia has been problematic because normal animals do not appear to have a sustained response to LPS administration. Repeated boluses of endotoxin and chronic endotoxin infusion both failed to produced a sustained cardiopulmonary response in sheep.[216,217] Horses given a 4 h continuous infusion of endotoxin responded in a similar fashion as those given endotoxin by IV bolus.[20] Horses given multiple intraperitoneal doses of endotoxin had similar hematological and metabolic responses as those given LPS by IV bolus.[85] This transient refractoriness to the effects of endotoxin (tolerance) has been recognized in healthy human beings and animals given repeated non-lethal doses of LPS or during chronic LPS infusion.[218,219,220] It is interesting that normal animals become tolerant to the effects of continued exposure to endotoxin while endotoxemic patients appear to have persisting LPS-induced effects such as circulating TNF activity, pyrexia, and hematologic alterations.[202,203]

A common apprehension about the clinical relevance of agents tested in a bolus model of endotoxemia is the necessity for treating prior to endotoxin exposure to show an effect. Potential therapies tested in this model must achieve appropriate circulating concentrations at time the animal responds to LPS administration. Studies where monoclonal antibodies against TNF were given prior to, or within an hour after, LPS was given were effective because the treatments were present at the time of TNF release. Since circulating TNF activity has correlated with outcome of clinical cases, it is reasonable to assume that anti-TNF antibody could be used therapeutically, particularly if cases with detectable circulating TNF activity could be quickly identified and targeted for anti-TNF treatment. The long half-life of these antibodies indicates that a single treatment may be protective in cases at risk for ongoing cytokinemia.

Important to future clinical application of anti-TNF antibody is the development of ways to identify quickly patients that are at risk for ongoing cytokinemia. Ideally, anti-TNF treatment would be evaluated in prospective, double-blind clinical trials of patients shown to have circulating TNF activity. Monoclonal antibodies against TNF would be appropriate reagents to use in developing an immunoassay to rapidly detect circulating TNF activity. Such TNF immunoassay could then be used to identify patients

for anti-TNF treatment, or to further investigate circulating TNF activity of endotoxemic patients and develop a better understanding of types of patients at risk for ongoing cytokinemia.

The clinical use of anti-TNF antibodies would need to be justified economically. The cost of producing and purifying 100 mg of antibody using hollow-core tissue culture technology should be around $50 - $75 dollars.[a] With minimal mark-ups, this amount of antibody could be made available to patients for $150 - $200. Giving 100 mg antibody to a 45 kg neonate should provide 2.2 mg antibody per kg body weight. As discussed in chapter 5, antibody dosages of 2 mg/kg should be more then adequate to neutralize circulating TNF activity in clinical cases. Treatment of critically-ill foals with anti-TNF appears to be justified economically when current intensive care costs of $200 - $300 per day[b] are considered. If anti-TNF treatment could shorten the period of intensive care by just one day it would be cost-efficient. Septic foals appear to be most suited for clinical trials using anti-TNF antibody. Their size would minimize cost of antibody treatments, they

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[a]Personal communication with Linda Green of the University of Florida ICBR Hybridoma Facility, Gainesville, FL.

[b]Personal communication with Dr. Guy Lester, Director of the Equine Neonatal Intensive Care Unit (ENICU), University of Florida, Gainesville, FL.

are often presented early during the course of disease, and equine neonatal sepsis is well-defined.[8,11]

There is a still a great deal to be learned about equine endotoxemia. Much of what is known has been extrapolated from results obtained in normal horses given LPS. The time-course of inflammatory mediator release, cardiovascular responses, and metabolic changes have not been studied extensively in equine endotoxemic patients. Why do normal animals become tolerant to endotoxin while endotoxemic patients suffer from continuing LPS-effects? What is the role of TNF binding proteins in protecting against the effects TNF production and could circulating TNF activity result from decreased amounts or impaired function of soluble TNF receptors, rather then from excessive TNF production? Hopefully, future studies will uncover mechanisms of dysregulated host responses to endotoxin, and provide further insight on treating endotoxemic patients.

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BIOGRAPHICAL SKETCH

Jana Lea Cargile was born on June 7, 1962, in Midland, Texas to Leslie Lee Cargile and Phyllis Ann Harp Cargile. Les Cargile worked in the overseas exploration division of the Mobil Oil Corporation, and the Cargile family lived in Lagos, Nigeria, then Medan, Indonesia, from 1968 to 1975. The family returned to the United States in 1975, and Jana attended high school in Grapevine, Texas; Covington, Louisiana; Wilton, Connecticut; and Houston, Texas.

Jana received the Bachelor of Science and Doctor of Veterinary Medicine Degrees from Texas A&M University in 1985 and 1987, respectively. She then completed a rotating equine internship at the University of Missouri, Middlebush Equine Center in 1988. In 1988, Jana joined the University of Florida, Large Animal Clinical Sciences Department as a Large Animal Medicine Resident. After completing the two-year residency program, Jana entered the UF CVM graduate program to pursue a Ph.D., working under the supervision of Dr. Robert J. MacKay as a Morris Animal Foundation Fellow. During her graduate studies, Jana served as treasurer, then president of the UF Veterinary Graduate Student's Association.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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